

2011

Evaluating the use of Flow-Through Larval Culture for the Eastern Oyster, *Crassostrea virginica*

Stephanie L. Reiner

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<https://dx.doi.org/doi:10.25773/v5-2s4b-k150>

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Evaluating the use of flow-through larval culture for the Eastern oyster,
Crassostrea virginica

A Thesis

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

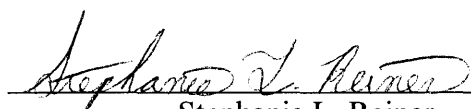
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Stephanie L. Reiner


2011

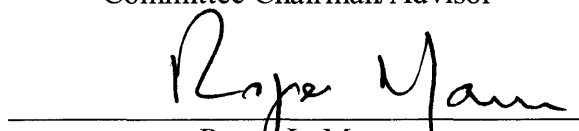
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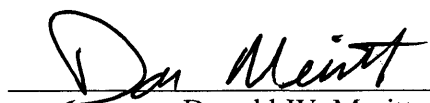
This thesis is submitted in partial fulfillment of
the requirements for the degree of
Master of Science


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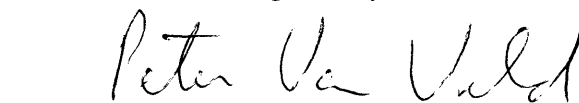

Peter A. Van Veld

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ACKNOWLEDGEMENTS

I would first like to thank my advisor, Stan Allen, for his guidance and support throughout the course of my graduate career. Thanks to my committee, Roger Mann, Pete Van Veld, and Don Meritt, for your many ideas and suggestions on how to make this project a success.

This project required a lot of larvae, and so required many hands to help with spawning. I would like to thank the entire ABC crew for all of your help in the hatchery, in the lab, and in the field. Thank you Katie Blackshear, Brian Callam, Amanda Chesler, Linda Crewe, Adeline Fortin, Anu Frank-Lewale, Nate Geyerhahn, Karen Hudson, Kate Ritter, Karen Sisler, and Erin Schramski. Whether I needed to do an emergency spawn or was having trouble in the lab you guys always came through. The crew at the Horn Point Lab also deserves thanks for providing me with ripe brood stock and larvae. Howard Kator and Martha Rhodes advised me on the TCBS portion of this project and allowed me the use of their lab to make my plates. Thanks also to Jordan Snead for helping me set up my flow-through system and to Shaq for enduring the heat and humidity to help me drain and sample my tanks.

Without the help and time of the hatchery trio at Oyster Seed Holdings this project wouldn't have existed. Thanks to Mike Congrove for giving me my corner of hatchery space and to Sean for delivering countless buckets of algae. A special thanks to my oyster mama and personal cheerleader, Judy Ambrose, for providing laughter through the hottest and hardest parts of summer.

Three years of grad school were made easier with the strong support from my friends here at VIMS and elsewhere. Thank you Carolina Funkey, Brenna Higgins, Clare McLean, Emily Nelson, Gina Ralph, Stephanie Salisbury, Gabby Saluta, Amy Then and Laura Wallace for encouraging me, lending an ear when I needed one, and ensuring I got out for fun every once in awhile.

My mom and dad gave me everything I needed to succeed at life, as well as grad school, and I thank them for 26 years of patience, hard work, and love. Allison and Carolyn, thanks for always being on my side (even if it was difficult at times) and for the many inside jokes.

Last, but certainly not least, a heartfelt thanks to David Marsich, my best friend and husband, for the wall of love and support I braced myself on time and again. You always managed to reduce my frustrations to their proper size and never failed to make me smile.

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GLOSSARY

Competent (pediveliger) – the last stage of larval development, characterized by the appearance of a pigmented “eye” spot and a crawling foot, indicating the larva is ready to undergo metamorphosis. In aquaculture, larvae are harvested from their cultures once the competent stage is reached

D-stage – the early stage of larval development whereby the shell is formed and larvae have a “D”-shaped appearance

Downweller – a growing system used to rear post-metamorphic juvenile oysters where water flow enters from the top of the growing container

Duration of competent larvae – a metric developed for this thesis that describes the number of days between and including when 10% and 90% of the competent larvae have been harvested from a culture

Metamorphosis – irreversible process of the oyster life cycle that describes the transition from the pelagic larval stage to the benthic juvenile stage

Peak harvest – used in this thesis to refer to the day when the majority of the competent larvae are removed from a flow-through culture

Set (setting, settle) (term used to describe settlement and metamorphosis) – all-encompassing word to describe the process of larval settlement to a substrate, exploration of that substrate, and metamorphosis to the juvenile stage

Settlement – reversible process where the larva makes contact with and explores a substrate prior to undergoing metamorphosis

Upweller – a growing system used to rear post-metamorphic juvenile oysters where the water flow enters from the bottom of the growing container

Veliger –the period of the larval cycle between the D-stage and competent stage

ABSTRACT

One system used for bivalve mollusc culture is flow-through larval culture, which provides a continuous flow of food and seawater to the tank. Flow-through culture enables larvae to be reared at stocking densities up to 100 larvae/mL, a characteristic that should recommend it as the culture system of choice for the East coast; however, Eastern oyster larvae have never been tested in flow-through culture, discouraging implementation of the system. The thesis objectives are designed to address questions regarding the survival, growth, competent period, cell consumption, growth efficiency, and cell selection of oyster larvae reared in flow-through culture. The objectives are: to describe larval tolerance to metabolic waste products, to determine how stocking densities influence clogging of the banjo screen and how those stocking densities coupled with exchange rate influence survival, growth, duration of the competent period, cell consumption, and cell selectivity, and to examine replication of flow-through culture and establish data for the variables measured. To obtain a basic understanding of larval tolerance to their metabolic waste, twelve static tanks were set up at the Aquaculture Genetics and Breeding Technology Center's hatchery at the Virginia Institute of Marine Science. Larvae were exposed to a range of concentrations of ammonia, nitrite, and nitrate and their survival and growth were monitored. Ammonia was the only metabolic waste that caused detrimental effects to larvae at a concentration of 10 mg/L. To address the remaining objectives, six 400 L conical flow-through tanks were set up at Oyster Seed Holdings, a commercial hatchery. To determine if banjo screen clogging (the cause of tank overflow) was affected by the day and density at which larvae were introduced to flow-through culture, larvae were introduced at two days old at three different stocking densities. The banjo screen, a circular plastic band with mesh screen on both sides, retains larvae in flow-through culture while allowing water to exit. The banjo screen was monitored for clogging every 12 hours for 60 hours. Larvae can be introduced to flow-through culture at two days old at densities as high as 50 larvae/mL without risking banjo clogging. To examine the effects of different flow-through culture parameters on larval development, larvae were stocked in flow-through cultures at 10, 20, and 50 larvae/mL and reared at five and ten exchanges of water/day. Five exchanges of water/day and a stocking density between 10 – 20 larvae/mL resulted in the highest survival, fastest growth, and greatest amount of competent larvae harvested. Variation among flow-through cultures stocked with 10 larvae/mL and reared at five and ten exchanges of water/day was examined. Five exchanges of water/day generally had lower variation, with the smallest being survival and length. The results for survival, length, cell consumption, duration of the competent period, and growth efficiency were characterized as the established values for the thesis's flow-through system and were compared with data obtained from the controls in Chapter Three to distinguish anomalous data.

Evaluating the use of flow-through larval culture for the Eastern oyster, *Crassostrea virginica*

Chapter One: Introduction to oyster aquaculture and the flow-through system

Introduction

History of Oyster Aquaculture

The increase in human population and the resultant increase in demand for seafood products are straining natural fisheries and impelling aquaculture development, which experienced explosive growth during the twentieth century. Total production from aquaculture was less than a million tons in the 1950s, and increased to 51.7 million tons in 2006 (47% of the total global seafood harvest) (FAO, 2008). The total encompasses a wide range of seafood species, including shellfish (Stickney, 2005). By 2000, global bivalve production from culture operations was 14 million tons, accounting for 75% of the total global harvest (Helm et al., 2004). In Virginia, sales of market-size *Crassostrea virginica* produced via aquaculture (now up to about 17 million per annum) increased 50% from 2007 – 2008, 26% from 2008 – 2009, and a further 34% from 2009 – 2010 (Murray and Oesterling, 2010; Murray and Hudson, 2011). Although industry growth is recent, it represents the product of an accumulation of research and technological developments spanning decades.

One important scientific contribution to Eastern oyster aquaculture occurred in 1879 when the life cycles of the European flat oyster, *Ostrea edulis* and the American oyster (now known as the Eastern oyster, *C. virginica*) were differentiated (Brooks, 1879). “Common knowledge” at the time stated that egg fertilization and larval development of these two species were the same: fertilization and brooding of the young

larvae occurred in the mantle cavity of the female and larvae were eventually released into the water column as veligers. The misinformation was corrected by Brooks (1879), who discovered that fertilization and larval development of the Eastern oyster occur in the water column. Successful egg fertilization and subsequent survival of the larvae in a laboratory setting proved the species was amenable to aquaculture, while the discovery of the mechanism of fertilization provided an important contribution to understanding the life history of the Eastern oyster.

Once Eastern oysters could be successfully spawned in an aquaculture setting, a method was needed for separating the minute larvae from the water column. The inability to retain larvae made water changes and consequently natural phytoplankton replenishment – the main food source of the larvae at this time – impossible (Wells, 1920). Centrifuging was the first method used for separating larvae from seawater. The newly-formed shell of the D-stage provided larvae with protection as they were spun and collected in the bottom of a vial, allowing transfer of the larvae to a new tank. Centrifuging also provided a means for collecting samples of larvae, so the entire larval life cycle could now be documented (Wells, 1920).

The larval cycle of the Eastern oyster can last two to three weeks; exact development time is influenced by environmental conditions (temperature, salinity, food quality, etc) (Galtsoff, 1964; Castagna et al., 1996). Gametes are released into the water column where fertilization occurs and within 24 hours the egg hatches into the trochophore stage and progresses to the D-stage. Within another 24 hours the larva transitions into the veliger stage, when the digestive system, velum, and umbone develop. Larvae continue to grow during this time, and when they have reached a length of at least

300 μm , a pigmented “eye spot” develops along with a crawling foot signifying that the larva is ready to settle and metamorphose to the juvenile stage. After exploring the substrate and cementing its left valve to the chosen spot, the larva undergoes metamorphosis and enters the sessile phase of the life cycle. The larva will remain in the chosen spot for the remainder of its life, continuing to grow and reaching maturity at approximately a year old (Galtsoff, 1964; Thompson et al., 1996; Helm et al., 2004).

W. F. Wells, from the New York Conservation Commission, working at his lab on the shore of Great South Bay, NY, filed for a patent in 1922 on the methods of oyster culture. The patent was approved in 1933 (Wells, 1933). Further research on the larval life cycle provided descriptions of the structure and function of larval organs and the morphological changes that occurred during metamorphosis (Prytherch, 1934; Galtsoff, 1964). The information obtained from this research encouraged the filing of a second patent, approved 30 years after the first, which included revised larval culturing methods as well as instructions on how to raise seed (juvenile) oysters (Glancy, 1965). Other advances in oyster aquaculture included the replacement of centrifugation with mesh screens that could be used to retain larvae during tank draining, and a change in diet from natural phytoplankton brought in from the outside seawater source (Wells, 1933) to a diet of cultured algae grown in the hatchery. Research into cultured algae determined that a mixed species diet was better for larval growth than a single species, so hatcheries began growing a variety of algal species in their facilities (Bruce et al., 1940; Loosanoff and Davis, 1963; Brown et al., 1997). Some of the most commonly cultured algal species are *Tetraselmis suecica*, *Isochrysis* sp., *Pavlova lutherii*, *Chaeoceros* sp., and *Thalassiosira pseudonana* (Helm et al., 2004).

Larval Culturing Systems

One system used for rearing oyster larvae is known as the static system, which requires a standing tank of seawater to culture the larvae, with draining and cleaning of tanks occurring every 2 – 4 days (Castagna et al., 1996, Helm et al., 2004). Procedures for rearing larvae in static culture recommend stocking tanks with 4 – 15 D-stage larvae/mL and feeding cultures once or twice daily via addition of algae (Loosanoff and Davis, 1963; Helm and Millican, 1977; Castagna et al., 1996; Robert and Gérard, 1999; Helm et al., 2004). The feeding densities of algae can vary between 15,000 – 100,000 cells/mL depending on the age, size, and density of the larvae, but this method of feeding lacks precision (Davis, 1953; Malouf and Breese, 1977; Helm et al., 2004). When the daily ration is added to a culture, larvae are initially overfed. Then, as the larvae gradually graze on the algae and reduce its density, there is the potential for larvae to be underfed for the remainder of the day (Malouf and Breese, 1977).

Another system for rearing oyster larvae is the flow-through system. One such system, described by Southgate and Ito (1998) was labeled a partial flow-through system, meaning that water flow was shut off during the day, allowing larvae to feed, and resumed at night. Flow-through systems can also be described as continuous, meaning that water and food are constantly supplied to the tank. Continuous flow-through systems have been used to successfully culture scallops, oysters, lobsters, mussels, shrimp, and several species of fishes (Malouf and Breese, 1977; Robert and Gérard, 1999; Andersen et al., 2000; Baskerville-Bridges and King, 2000; Ritar, 2001; Tieman and Goodwin, 2001; Otoshi et al., 2003; Sarkis et al., 2006; Rico-Villa et al., 2008).

There are several methods for constructing a flow-through system, but all require some mechanism for larval retention as well as a means to provide continuous food and water flow to the tanks. The most common method of larval retention is a banjo screen that fits over the outflow pipe, allowing water and waste products to exit. As the larvae grow, the banjo screen (Fig. 1.1) can be exchanged for one with a larger mesh size (Andersen et al., 2000; Ritar, 2001; Rico-Villa et al., 2008). The larger mesh on the banjo screen enhances water flow through the tank, reducing the likelihood of banjo clogging and larvae becoming impinged on the banjo screen. The constant replenishment of clean water and food characteristic of flow-through systems can support a greater stocking density: 15 – 100 larvae/mL, an increase ranging from 1 – 25 times greater than the 4,000 – 15,000 larvae/L maintained in a static system (Loosanoff and Davis, 1963; Malouf and Breese, 1977; Castagna et al., 1996; Helm et al., 2004; Rico-Villa et al., 2008).

Flow-through culture: Oyster larvae

The Pacific oyster, *Crassostrea gigas*, is one species that has been reared using continuous flow-through larval culture. One flow-through system used to raise *C. gigas* larvae used 150 L cylindro-conical fiberglass tanks (Rico-Villa et al., 2008). Seawater mixed with algae entered the tank from the top at a flow rate of 0.66 L/min, equivalent to six exchanges of water/day. “Exchange” refers to the complete replacement of the volume of seawater in a tank with a new supply of seawater. Water flowed out of the system via a submerged rectangular sieve covered with nylon mesh for larval retention. The mesh sizes were 40, 60, and 80 μm . As larvae grew, the mesh size was increased to improve water flow. An aeration line was connected to the bottom of the tank. The

outflow from the tank accumulated in a 70 L “reservoir” and was then pumped through a sieve (size not provided in paper) to remove debris before being measured for temperature, pH, salinity, and fluorescence. The water quality measurements were performed automatically six to seven times a day and allowed the researchers to monitor water quality and feeding without disturbing the larvae (Rico-Villa et al., 2008).

In the flow-through system described above, tanks were stocked with two-day-old (D-stage) larvae at 5, 50, and 100 larvae/mL and reared at a temperature of 25°C. There was no significant difference in larval length to day 16 (when larvae became competent) between the 50 and 100 larvae/mL stocking densities. Additionally, there was no significant difference in survival or metamorphosis between the stocking densities of 50 and 100 larvae/mL. Reported survival to day 16 was over 90% for stocking densities of 50 and 100 larvae/mL. To evaluate metamorphic success (the number of competent larvae that successfully completed metamorphosis), Rico-Villa et al. (2008) placed plastic disks in the flow-through cultures to collect metamorphosing larvae. After four days, the number of larvae in the flow-through culture that had not metamorphosed was subtracted from the total number of larvae initially stocked in the flow-through culture. The results were confirmed by estimating the number of juvenile oysters attached to collectors and tank walls. The stocking densities of 50 and 100 larvae/mL had over 80% of the larvae successfully undergo metamorphosis (Rico-Villa et al., 2008).

Interestingly, flow-through cultures stocked at 5 larvae/mL had significantly smaller shell length and significantly lower metamorphic success than larvae at stocking densities of 50 and 100 larvae/mL. It should be noted that while significant, the shell length and metamorphic success of the 5 larvae/mL stocking density treatment was only

marginally lower than that of the stocking densities of 50 and 100 larvae/mL. No explanation was provided for why the stocking density of 5 larvae/mL had the lowest growth and metamorphic success; instead the study focused on the success of rearing larvae at densities of 50 and 100 larvae/mL.

A second continuous flow-through design used for rearing Pacific oyster larvae used 6 L tanks constructed from a section of PVC pipe (76 cm long, 10 cm in diameter) (Malouf and Breese, 1977). Only the cultures of algae, not seawater, constantly flowed into the tanks. The flow of algae was at a rate of 0.014 L/min, equivalent to about one exchange of water/day. A mesh screen was fitted over the outflow to retain larvae and an aeration line was connected to the bottom of the tank. The Malouf and Breese (1977) system explored the affects of different inflow densities of algae on different larval stocking densities starting when the larvae were approximately two weeks old. Higher inflow rates of algae increased the growth rate of larvae. Under equal inflow densities of algae, larvae in tanks stocked at 2 larvae/mL had faster growth rates than larvae stocked at 16 larvae/mL.

The Malouf and Breese (1977) system experienced several problems that may have affected larval growth. In one experiment, larvae were exposed to “cooler” temperatures than larvae in subsequent experiments, which may have influenced growth; however, data for temperature were not provided, so it is uncertain to what extent temperatures were “cooler”. Malouf and Breese (1977) note that the larvae experienced an increase in growth rate with an increase in temperature, so the assumption is that “cooler” temperature inhibited growth of larvae. Only one species of algae, *Monochrysis lutheri*, was fed to the larvae.

The Malouf and Breese (1977) flow-through system was constructed on a smaller scale than other experiments involving flow-through larval culture. The Malouf and Breese (1977) flow-through tanks had a 6 L volume while Rico-Villa et al. (2008) used 150 L tanks. Other studies discussed later in this section used flow-through tanks with volumes of 500 L (Southgate and Ito, 1998; Andersen, 2000) and 4700 L (Andersen, 2000). Finally, the Malouf and Breese (1977) flow-through cultures did not have continuous seawater flow other than the algae cultures themselves.

A partial flow-through system was used to culture *Pinctada margaritifera*, pearl oyster, larvae and used 500 L fiberglass tanks (Southgate and Ito, 1998). A flow rate of 0.83 L/min, equivalent to one exchange of water/day, entered the tank from the top. A standpipe covered in 37 μ m mesh was situated in the middle of the tank to retain larvae. Aeration was provided through the bottom of the standpipe. The Southgate and Ito (1998) system differs slightly from those already mentioned in that it was set up as partial flow-through, meaning that seawater flow was shut off for 12 hours when algae were introduced to the culture, allowing larvae time to feed. This thesis used the continuous method of flow-through culture, but partial flow-through culture is mentioned here to provide an example of another method for flow-through larval culture.

The objective of the Southgate and Ito (1998) study was to determine whether pearl oyster larvae could be reared under partial flow-through culture conditions. D-stage (day 2) larvae were introduced to the culture at 1 larva/mL. Survival gradually declined during the culture period; by day 23, when the first competent larvae were removed for metamorphosis, survival was 11.3%. Survival was comparable to other studies performed on pearl oyster larvae reared in static systems (Southgate and Ito, 1998). The

partial flow-through system was considered a success for rearing pearl oyster larvae because while the results were similar to those obtained from a static system, the partial flow-through system provided other benefits, such as reduced labor, reduced handling of larvae, and (the study assumed) better water quality due to more frequent water exchanges (Southgate and Ito, 1998).

Flow-through culture: Scallop larvae

Argopecten gibbus (calico scallop) and *Pecten maximus* (great scallop) larvae are two species of scallop that have been cultured in a continuous flow-through system (Sarkis et al., 2006; Magnesen et al., 2006). Sarkis et al. (2006) cultured calico scallop larvae in 200 L conical flow-through tanks. An upwelling system was implemented to introduce seawater and aeration into the tank. A 10 L bucket served as a head tank to maintain a constant water flow, and it was from here that seawater was drawn for the flow-through tank. A pipe connected to the bottom of the head tank allowed water to flow by gravity down the pipe and into the bottom of the larval tank. A one-way ball valve, a valve in which a ball moves in and out of a socket in response to changes in pressure, regulated water flow at a rate of 0.48 L/min, equivalent to 3.4 exchanges of water/day. Banjo screen sizes ranging between 40 – 80 µm retained larvae within the flow-through culture. The daily allowance of algae was divided into three rations. The first ration was added manually to the tank at once while the other two rations were drip-fed to the culture over 24 hours.

Two stocking densities were used by Sarkis et al. (2006): 8 and 24 larvae/mL. Larvae were introduced to the flow-through tanks when they were two days old. During the time larvae spent in culture they were fed between 7,000 – 21,000 cells/mL daily.

From day 8 to day 13 the stocking density of 24 larvae/mL had the highest survival, yet growth of larvae stocked at 24 larvae/mL was slower than the growth of larvae stocked at 8 larvae/mL. Metamorphic success rate was lower and juvenile oysters were smaller at a stocking density of 24 larvae/mL compared to a stocking density of 8 larvae/mL. Sarkis et al. (2006) speculated that slower growth and poorer metamorphic success observed at a stocking density of 24 larvae/mL were due to increased competition for food that lowered the energy reserves available for growth and metamorphosis.

A second continuous flow-through system used by Magnesen et al., (2006) to rear scallop larvae used 3500 L conical tanks provided with a flow rate that resulted in one exchange of water/day. Mesh screen 60 μ m in size was used initially to retain larvae in the tank, and then replaced with 80 μ m screen. Algae were added to flow-through cultures to attain densities between 10,000 – 20,000 cells/mL. Larvae were introduced to the system when they were three days old at four different stocking densities: < 4 , $\geq 4 - 6$, $\geq 6 - 8$, and > 8 larvae/mL. No significant difference in growth or survival of larvae was observed among the four stocking densities. The two lowest stocking densities (< 4 and $\geq 4 - 6$ larvae/mL) had greater metamorphic success than the two highest stocking densities ($6 - 8$ and > 8 larvae/mL) (Magnesen et al., 2006).

Andersen et al., (2000) examined continuous flow-through culture of scallop larvae using 500 L conical downwelling tanks and one 4700 L conical upwelling tank. The 500 L downwelling tanks had seawater and algae entering from the top and exiting at the bottom of the tank. The flow rate was set to provide one exchange of water/day. The bottom of the tank contained a mesh sieve sized to retain the larvae. The size of the mesh was 63, 80, or 100 μ m in size, depending on the size of the larvae. Algae were added to

the tank to achieve a density of 50,000 cells/mL; afterwards the flow rate was set to maintain an inflow density of 25,000 cells/mL. The 4700 L upwelling tank had seawater entering from the bottom. Seawater flow was set to provide one exchange of water/day and seawater exited the tank through a mesh-covered sieve at the top. The sieve size was increased as the larvae grew. Algae were added to achieve a density of 25,000 cells/mL in the tank; afterwards the flow rate was set to maintain an inflow density of 25,000 cells/mL. Aeration for the 500 and 4700 L tanks entered from the bottom.

The Andersen et al., (2000) system was designed to test whether flow-through culture reduced the number of bacteria colonies observed in the system when compared with two static tank treatments: one that had been treated with antibiotics and one that had not. Final results indicated that the 500 and 4700 L flow-through tanks had fewer bacteria colonies than both the treated and untreated static tanks.

Conclusions

In terms of setting up flow-through larval culture, whether partial or continuous, all of the studies mentioned above used conical tanks with some form of aeration entering from the bottom. In all instances, larvae were retained in culture vessels by a mesh screen ranging between 40 – 80 μm in size that was increased to correspond to increases in the size of larvae. Whether the seawater entered from the bottom or the top of the tank, the flow rate that was most commonly used was equivalent to one exchange of water/day for cultures stocked between 1 – 16 larvae/mL (Malouf and Breese, 1977; Southgate and Ito, 1998; Magnesen et al., 2006). When a culture was stocked at higher densities, 50 – 100 larvae/mL, a flow rate that allowed for six exchanges of water/day was used (Rico-Villa et al., 2008). This thesis examined high density culture in a

continuous flow-through system that mimics that of an operating commercial hatchery, so a higher exchange rate was used.

The studies discussed in the introduction demonstrated that larvae of their respective species could be reared successfully in flow-through culture, but flow-through culture conditions used to rear larvae varied among species. There is no documentation in the literature discussing the rearing of *C. virginica* in flow-through larval culture, so this research examined a previously untested organism.

The studies discussed above introduced larvae to flow-through culture when they were two or three days old at different stocking densities. Survival, growth, and metamorphic success differed depending on stocking density. For Pacific oyster larvae, stocking densities of 50 and 100 larvae/mL did not impair survival, growth, or metamorphic success. For scallop larvae reared in flow-through culture, increasing the stocking densities impaired metamorphic success and growth of larvae.

The results obtained from flow-through culture studies involving *C. gigas*, *P. margaritifera*, *A. gibbus*, and *P. maximus* larvae were unique to each species. Flow-through culture results of one species could not have been used to predict flow-through culture results of another species, and so cannot be used to predict how flow-through culture will affect the development of *C. virginica* larvae. From the previous studies, it is apparent that larvae of different species respond in distinctive ways to the techniques of flow-through culture. Each species requires its own trial to determine how various methods of flow-through culture will affect larval development.

Continuous flow-through larval culture for *C. virginica* has only recently been adopted. Two hatcheries, Oyster Seed Holdings, LLC (OSH) and Kellum, Cowart, and

Bevans (KCB) were recently built in Virginia under consultation by Jim Donaldson, formerly the hatchery manager for Coast Oyster Company in Quilcene, Washington (1974-2000), now known as Coast Seafoods Company. At Coast Oyster Company, Donaldson developed techniques for rearing *C. gigas* and *Ruditapes philippinarum*, the Manila clam, in flow-through culture. Under his guidance, OSH and KCB adopted flow-through culture for oyster larvae. To better inform these and other hatchery managers on the development of Eastern oyster larvae in flow-through culture, factors that influence larval development, such as, stocking density and water exchange rate need to be assessed. Once the impacts of these parameters on survival, growth, and development of larvae have been examined, managers should be able to make informed decisions regarding the application of this system to their own hatcheries.

Objective

The first objective of this thesis was to determine the effects of selected metabolic waste products on oyster larvae stocked at high density (10 – 50 larvae/mL) in a continuous flow-through culture.

The second objective was to determine how stocking densities of 10, 20, and 50 larvae/mL, influenced clogging of the banjo screen at screen sizes of 41 and 60 μm and how those same stocking densities coupled with exchange rates of five and ten per day influenced survival, growth, duration of the competent period, cell consumption, and cell selectivity of larvae.

The third objective of this thesis was to examine the replication of flow-through culture in the system used for this thesis and establish data for the variables measured.

The established data were used as a basis to compare results obtained from the second objective.



Figure 1.1. A flow-through tank with a detachable banjo screen attached to the outflow pipe.

Chapter Two: Flow-through culture of oyster larvae: Retention of larvae in culture tanks and tolerance to metabolic waste

Introduction

Typically, the operating procedure for rearing bivalve larvae in static culture is to introduce eggs to the tank on the day of fertilization and maintain tank cleanliness by draining and cleaning static cultures every 2 – 4 days (Castagna et al., 1996; Helm et al., 2004). These procedures are designed to maintain the health of the larvae. In the context of the commercial hatchery, Oyster Seed Holdings (OSH), where this research was accomplished, eggs destined for flow-through culture are first held in a separate incubation tank before introduction to flow-through culture. Cleanliness in flow-through tanks is maintained by filtered seawater flowing through the tank in addition to periodically draining and cleaning the tank. Some flow-through cultures also require maintenance of the banjo screen and regulating water inflow into the tank. The maintenance requirements necessary to prevent clogging of the banjo screen for the flow-through system of this thesis were examined. The tolerance of larvae to nitrogenous compounds related to their metabolic waste was also examined.

Retention of larvae in culture vessels

Introduction of larvae to flow-through culture can occur at any stage of larval development. Larvae of Pacific oyster, pearl oyster, and calico scallop were introduced to flow-through culture as young as two days old (Southgate and Ito, 1998; Sarkis et al., 2006; Rico-Villa et al., 2008). Great scallop and Pacific oyster larvae have been

introduced to flow-through culture as old as one to two weeks of age (Malouf and Breese, 1977; Magnesen, 2006).

The commercial hatchery, OSH, where this research was conducted experienced problems with tank overflow during the first week of larval development in flow-through culture during the summer of 2009. The size of the larvae at this age required the use of a small mesh size on the banjo screen (41 μm) to ensure retention of larvae. Personnel at OSH hypothesized that the small mesh combined with poor algal quality during the first week of larval development resulted in clogging of the banjo screen and overflow of the tank. To maintain cleanliness and prevent overflow of the tanks, personnel at OSH were required to clean the banjo screens multiple times per day. As a solution, OSH delayed the introduction of larvae to flow-through culture until they were four to six days old, when larger mesh (60 μm) could be used on the banjo screen. Clogging of the banjo screen was an unanticipated logistical problem and provided another aspect of flow-through culture that needed to be addressed: what conditions of flow-through culture can result in clogging of the banjo screen and overflow of the tank?

Tolerance to metabolic waste

To maintain a healthy culture, metabolic waste must remain at levels that larvae can tolerate, but very little is known regarding the tolerance of *Crassostrea virginica* larvae to their metabolic waste. Ideally, the exchange rate of water in the flow-through tank should be fast enough to remove metabolic waste, but slow enough to provide larvae with a chance to feed. Since flow-through systems have a constant replenishment of algae (Stickney, 1994; Helm et al., 2004) that enables them to support higher stocking densities of larvae, there is a constant opportunity for larvae to feed. The constant

opportunity to feed combined with the increase in larvae that are producing metabolic wastes results in a greater risk for accumulation of metabolic waste that could potentially poison the larvae.

The main excretory product of marine invertebrates is ammonia, which can oscillate between ammonia and ammonium depending on pH; acidic conditions favor ammonium and basic, ammonia (Warren, 1962; Campbell, 1973). At OSH the pH ranged from 7.24 – 8.29, conditions that favor ammonia. Ammonia can be oxidized to nitrite, and then to nitrate via nitrification as part of the nitrogen cycle (Colt and Armstrong, 1981; Stickney, 1994; García-Esquivel et al., 2001). Excretion rates for Pacific oyster larvae range from 0.245 – 1.638 mg NH₄-N/larva/hr, so it is likely that the excretion rates for Eastern oyster larvae fall near this range (García-Esquivel et al., 2001).

Exposure to high concentrations of ammonia, nitrite, and nitrate affects aquatic organisms in a variety of ways. Ammonia concentrations greater than 0.021 mg/L in a culture system can cause gill damage, which impairs oxygen transport to the tissues, and a reduction in feeding that ultimately reduces growth of the organism (Colt and Armstrong, 1981). Nitrite concentrations greater than 16 mg/L can affect water quality by causing a decline in pH (Collins et al., 1975). Low pH can be harmful to *C. virginica* larvae, which require a pH range of 6.75 – 8.75 for normal development. A pH of 6.75 can result in increased mortality, reduced shell growth and calcification, and a reduction in shell thickness (Calabrese and Davis, 1966; Kurihara et al., 2007; Miller et al., 2009). Nitrite is rare in natural waters since bacteria quickly oxidize it into nitrate, but can be problematic in an aquaculture facility where high densities, limited bacteria growth, and inadequate filtration can promote its accumulation (Stickney, 1994). Nitrate, the least

toxic, rarely causes detrimental effects to a culture because the concentrations required are so high (greater than 23,030 mg/L for juvenile and adult oysters) it is unlikely they would ever be encountered (Epifanio and Srna, 1975).

In oysters, the most noticeable effects of toxicity from over-exposure to ammonia, nitrite, and nitrate involve valve function and filtration. Adult and juvenile oysters react to ammonia concentrations over 198 mg/L by keeping their valves closed, whereas prolonged exposure to nitrite concentrations exceeding 2600 mg/L has the opposite effect of inducing gaping. A reduction in clearance rate was observed under nitrate concentrations of 16,800mg/L (Epifanio and Srna, 1975). Accumulation of any of these metabolic waste products in the tank can increase bacteria numbers, alter the pH, or harm the development of the larvae (Calabrese and Davis, 1966; Helm et al., 2004).

Monitoring the rise of ammonia, nitrite, and nitrate may provide an indicator of culture health that could be used as a reliable method of monitoring cultures and a warning system indicating culture distress (in the form of mortality, inhibition of growth, or other developmental abnormalities). For flow-through culture, the tolerance levels of larvae to their metabolic waste can determine the exchange rate needed to prevent the accumulation of metabolic waste.

Methods

Retention of larvae in culture vessels

Set-up of the flow-through system

The experiment to address the conditions of flow-through culture that result in clogging of the banjo screen was conducted in April 2010 at OSH, located on Gwynn's Island in Mathews, Virginia (Fig. 2.1). At OSH, six conical 400 L flow-through tanks and two cylindrical 4700 L egg incubation tanks were installed. The six flow-through tanks were integrated into the OSH seawater, algae, and larvae rearing systems.

Sterilization of the OSH and thesis flow-through systems was performed once a week and involved pumping a 0.01% bleach solution through the seawater and algae delivery pipes and flow valves. Each flow-through tank was equipped with two separate flow valves, one for seawater and one for algae, to enable individual adjustment of seawater and algae flow to each tank (Fig. 2.2). Each flow-through tank was also equipped with an emergency overflow pipe connected to the banjo screen and ending at a Nitex screen ranging in mesh size from 50 – 63 μm , depending on the size of the larvae. The Nitex screen allowed for the recovery of any “spilled” larvae should the banjo screen clog and the tank overflow (Fig. 2.3). The banjo frames were constructed of circular PVC pipe, measuring 20.32 cm in diameter, 0.5 cm thick, and 7.62 cm in height. Six banjo frames were equipped with each of four Nitex mesh screen sizes. The four mesh screen sizes

were 41 μm (30% open area (OA)), 60 μm (42% OA), 100 μm (44% OA), and 150 μm (50% OA).

At OSH, algae are grown in 400 L polyethylene growing bags contained within wire cages. Carbon dioxide is supplied to each growing bag at a flow rate intended to maintain pH within a range of 7.8 – 8.1. Each growing bag has nine 40 watt lights that remain on 24 hours a day, except in summer when they are turned off in the afternoon to prevent an increase in room temperature. A commercial nutrient mix is added to 1 μm filtered and pasteurized seawater to make the media for growing algae. The media flows into the growing tubes at approximately 85 mL/min. Attached below the water line of each growing bag is a tube through which algae are continually harvested (Fig. 2.4). The harvest is collected in containers and then pumped to one of two larger holding tanks. One tank is designated for *Pavlova lutherii* and *Isochrysis* sp., and the second for *Chaetoceros* sp. and *Tetraselmis suecica*. Transfer of the algae to the respective holding tanks occurs constantly throughout the day. Once a day algae in the two holding tanks are combined into a large reservoir. From the reservoir, the algae are continuously pumped through the distribution system to the flow-through cultures. Unused algae are returned to the reservoir via a return pipe in the distribution system. The reservoir is large enough to supply larvae with food continuously, and the composition of algae in the reservoir varies from day to day.

Brood stock oysters, used to produce larvae for experiments on flow-through culture, consisted of wild oysters from the Chester River, MD, provided by the Horn Point Laboratory (HPL) in Maryland, and the genetically selected hANA line (high salinity-bred Louisiana oysters) reared at the Lynnhaven site by the Aquaculture Genetics

and Breeding Technology Center (ABC) of the Virginia Institute of Marine Science (VIMS). Chester River oysters were conditioned at HPL, acclimated to the salinity at OSH, and then strip spawned. Chester River oysters were only used for one experiment on flow-through culture; the remaining Chester River oysters re-absorbed their gametes before another spawn could be performed. The rest of the experiments on flow-through culture used the hANA line. Approximately 400 hANA oysters were conditioned at the ABC hatchery. Approximately one thousand hANA oysters were allowed to naturally condition at the ABC site in Sarah's Creek (a tributary of the York River) or the York River site behind VIMS. All oysters were strip spawned, a technique where oysters are shucked, the gonad is sliced to release gametes, and gametes are rinsed into beakers to await fertilization. Source of eggs was not considered a variable since the majority of the spawns used the same genetic line. When survival from the egg stage to D-stage (prior to introduction to flow-through culture) was examined, survival of larvae from the Chester River oysters and the hANA lines fell between 18 – 51%, suggesting little difference between wild and selected lines when hatched under similar conditions.

Stocking flow-through cultures and observations of banjo screens

Fertilized eggs were held in an incubation tank for 48 hours; after which larvae were introduced to the flow-through tanks. Two flow-through tanks were stocked at each of three stocking densities: 10, 20, and 50 larvae/mL and reared at a flow rate of 1.4 L/min (equivalent to five exchanges of water/day). It should be noted that the replicate tanks for the three stocking densities are replicating the ability of the operator to culture larvae in the flow-through system; they are not biological replicates. The mesh size of the banjo screen used on the day of introduction (day 2) was 41 μm . When larvae were 4

days old (a mean of 104 – 124 μm in length) the 41 μm banjo screen was replaced with a 60 μm banjo screen.

Introduction of larvae to flow-through tanks signified the beginning of a 60 hour observation period, divided into five observation blocks of 12 hours each. At the end of each observation block, note was made on whether clogging of the banjo screen had occurred and resulted in overflow of the tank. Banjo screens were cleaned at the end of every observation block and any live larvae expelled onto a catch screen due to overflow were examined and returned to the tank at this point. The observation period concluded when larvae were 108 hours (four and a half days) old. It should be noted that whenever the hour of an observation block is reported, it refers to age of the larvae, not the number of hours larvae spent in flow-through culture. For example, an observation made at 72 hours means larvae are 72 hours (three days) old, not that larvae have been reared in flow-through culture for 72 hours. Larvae were introduced to flow-through culture when they were 48 hours old, so at the 72 hour observation block larvae had been undergoing observations in flow-through culture for only 24 hours.

Metrics for larvae

Flow-through tanks were drained three times a week. Once drained, larvae were collected and rinsed through a series of decreasing screen sizes to determine which screen size retained the majority (>90%) of the population. A larger mesh size on the banjo screen would decrease the chances of clogging and overflow in this system, so when the majority of larvae were retained on a mesh size equivalent to the next largest banjo screen size (60 μm), the current banjo in the flow-through culture (41 μm) was replaced. The same procedure was followed for replacing any banjo screen with the next largest

size. After being rinsed through the decreasing screen sizes, all larvae were combined in a one or two liter beaker, depending on number of larvae. Larvae were counted from the beaker using a micropipette (Fisher Scientific, Pittsburgh, PA) that removed three 20 μ L samples from the beaker. Larvae in these three samples were counted under a microscope using a Sedgewick Rafter Counting Slide. Each count was multiplied by 50 to determine number of larvae/mL. Number of larvae/mL was multiplied by the volume of the beaker larvae were held in to determine the total number of larvae. Thirty-five larvae were killed with formalin and their shell lengths (longest dimension parallel to the hinge) (Galtsoff, 1964) measured to obtain mean growth rate. Flow-through tanks were rinsed with freshwater and cleaned with Scotch Brite scrubbing pads to remove fouling. The remaining living larvae were re-introduced to the clean flow-through tank once all measurements had been recorded.

Feeding

To ensure similar feeding opportunity within and among each flow-through culture, the outflow of each culture was regulated in such a way that cell density leaving each tank was the same, as determined by fluorometry. A FLUOROMETER (Turner Designs, Sunnyvale, CA) was used to maintain accurate densities of algae in flow-through cultures. Though less accurate than a Coulter Counter for determining density and composition of algae, the FLUOROMETER is more amenable to transport, rapid readings, and the hatchery environment. Each day, three samples of algae from the reservoir were diluted to the target density (25,000 cells/mL) by hemacytometer (Aquatic Eco-Systems, Apopka, FL) counts. Once the target density was obtained, the fluorescence of the sample was measured and mean fluorescence of the three samples

computed to provide the target fluorescence. The target fluorescence was compared to the fluorescence of water samples taken from the outflow of each flow-through culture. Adjustments were made to the flow rates for algae of each flow-through culture until the outflow fluorescence of the culture and the target fluorescence were the same.

Tolerance to metabolic waste

Set-up of the static system and determining concentrations for metabolic wastes

The experiment to determine tolerance of larvae to their metabolic wastes was conducted in August 2009 at the ABC hatchery at VIMS. Here, twelve, 57 L flat-bottomed static tanks were divided into three treatment groups for metabolic waste: ammonia, nitrite, and nitrate. Each treatment consisted of four tanks: one tank served as a control while the other three tanks were used to test response of larvae to different concentrations of the metabolic waste. Concentrations used for each treatment are given in Table 2.1 and were based on observations from static cultures reared before the experiment on metabolic waste was conducted, as explained below.

Prior to the experiment on tolerance to metabolic waste, observations were taken from a separate batch of larvae reared within a static system. Daily water samples were collected and analyzed for ammonia, nitrite, and nitrate. The highest observed concentration for each metabolic waste in the static trial served as the concentration tested in Treatment 1 for the experiment on metabolic waste. Concentrations used in Treatment 2 are double the concentrations observed in Treatment 1 (Table 2.1).

The third concentration of metabolic waste, Treatment 3, was intended to result in 100% culture distress, either in the form of complete mortality, impaired growth, or developmental abnormalities. Concentrations for Treatment 3 were estimated by using

data from Epifanio and Srna (1975). The concentration of ammonia that can be tolerated by juvenile Eastern oysters ranges from 110 – 880 mg/L (Epifanio and Srna, 1975).

Upon further examination of the Epifanio and Srna (1975) paper it was discovered that the reported 110 – 880 mg/L range for juvenile oysters was incorrect. Discussion with the primary author and subsequent re-calculation produced a new range of 198 – 1476 mg/L for ammonia. The 40 hour LC₅₀ concentration for Pacific oyster larvae exposed to ammonia was 0.6 mg/L (Wang et al., 1985). Therefore, the concentration that should cause 100% distress to Eastern oyster larvae was expected to range between 0.6 – 198 mg/L of ammonia. Oyster larvae most likely do not have as high a tolerance to ammonia as juvenile oysters, so testing concentrations as high as 198 mg/L seems excessive.

Supposing that Eastern oyster larvae would react to the effects of ammonia at similar concentrations as Pacific oyster larvae and assuming an exposure range for the biopsy of one order of magnitude, then the test range was estimated to be between 0.6 – 6 mg/L. The concentrations tested were 10 mg/L for ammonia and nitrite, and 20 mg/L for nitrate. The concentration for nitrate was greater than the concentration tested for ammonia and nitrite because nitrate is the least toxic metabolic waste of the three and should require a higher dosage to cause 100% distress to the larvae (Epifanio and Srna, 1975) (Table 2.1).

Once all static tanks for the experiment on tolerance to metabolic waste were filled with seawater, ammonia, nitrite and nitrate were added to their respective tanks. On water change days, metabolic wastes were added before larvae were re-introduced to the tanks. Temperature, pH, salinity, and dissolved oxygen were monitored using a HACH SensIon 156 Portable Multiparameter Meter (HACH Company, Loveland, CO). Larvae were fed once a day with 20,000 – 30,000 cells/mL of *Pavlova lutherii*.

Ammonia, nitrite, and nitrate were purchased from the HACH Company (Loveland, CO) as the following compounds: ammonium chloride (NH₄Cl), sodium nitrite (NaNO₂), and sodium nitrate (NaNO₃) based on protocols used in previous tolerance experiments on *C. virginica* (Epifanio and Srna, 1975). When calculating the quantity of each compound to add to obtain the experimental concentration, molecular weights of chloride and sodium were excluded from the calculations. An example of the calculations performed to determine the amount of compound to add to static cultures is provided below. The following calculation determines the concentration of ammonium chloride that should be added to static culture to obtain the target concentration of 0.15 mg/L for Treatment 1.

The first step converts the target concentration into amount of grams needed to achieve 0.15 mg/L in the entire 57 L tank:

$$\frac{0.15 \text{ mg}_{\text{NH}_3}}{\text{L}} * \frac{1 \text{ g}}{1000 \text{ mg}} * 57 \text{ L} = 8.55 \times 10^{-3} \text{ g}_{\text{NH}_3}$$

Next, the total amount of ammonia needed to achieve 0.15 mg/L is converted to moles using the molecular weight of ammonia:

$$0.00855 \text{ g}_{\text{NH}_3} * \frac{1 \text{ mol}_{\text{NH}_3}}{17 \text{ g}} = 5 \times 10^{-4} \text{ mol}_{\text{NH}_3}$$

Finally, ammonia was added to static culture in the form of ammonium chloride, so the molecular weight of ammonium chloride must be taken into account when calculating the final amount to be added:

$$5 \times 10^{-4} \text{ mol}_{\text{NH}_3} * \frac{1 \text{ mol}_{\text{NH}_4\text{Cl}}}{1 \text{ mol}_{\text{NH}_3}} * \frac{53.453 \text{ g}}{1 \text{ mol}_{\text{NH}_4\text{Cl}}} = 0.026 \text{ g}_{\text{NH}_4\text{Cl}}$$

Any concentration given in the text refers to concentrations of the target compound only: ammonia, nitrite, or nitrate.

Metrics for larvae

Fourteen female and seven male oysters from the ABC DBY06 Lynnhaven line (Delaware Bay oysters selectively bred for disease resistance at the Lynnhaven river in Virginia Beach, VA) were strip spawned to obtain eggs and sperm that were combined to obtain fertilized eggs. Eggs were divided among 12 tanks at approximately 50 eggs/mL and allowed to develop for 24 hours without exposure to metabolic wastes. Survival from egg to D-stage was between 27 – 43% among tanks. After 24 hours, tanks were drained onto 35 μ m mesh screens and larvae were counted. The larvae within each static tank were reduced to 570,000 larvae/tank, a density of 10 larvae/mL. Prior to returning larvae to their tanks, ammonia, nitrite, and nitrate were added to the respective treatment tanks.

Static tanks were drained every other day for one week. Larvae were caught on 35 – 60 μ m mesh screens (screen size increased as larvae grew) and counted to determine survival using the methods previously explained. Ten larvae were measured for length on a microscope with a calibrated ocular micrometer. Larvae were killed with formalin to enable measuring.

Measuring actual nitrogenous compounds

Water samples were taken twice daily, one hour before and after larvae were fed, to measure concentrations of nitrogenous compounds still in the water. A sample of fifty milliliters of water was removed from each tank, filtered through a 1.5 μ m filter disc using a hand-operated vacuum pump, and stored in bags on ice. Samples were analyzed

that same day with a HACH DR/890 Colorimeter according to the procedures outlined in the HACH DR/890 Colorimeter Procedures Manual (HACH Company, 2000). Each static tank, regardless of treatment, was analyzed for ammonia, nitrite, and nitrate. If the observed concentration of metabolic waste was found to be lower than the target concentration, more metabolic waste (in the form of the compounds purchased from HACH) was added to return to the proper testing concentration.

Results

Retention of larvae in culture tanks

Overflow did not occur in flow-through tanks stocked with 10 or 20 larvae/mL and reared using a flow rate of 1.4 L/min (equivalent to five exchanges/day). In the first flow-through tank stocked with 50 larvae/mL (50-A), overflow occurred at the observation blocks at 60, 72, 84, and 96 hours when a 41 μm screen (30% OA) was in use. At the observation block at 108 hours, the majority of larvae (>90%) had reached a size of greater than 85 μm and could be retained by a 60 μm screen. The 41 μm banjo screen was exchanged for a 60 μm screen (42% OA). No overflow occurred during the observation block at 108 hours when the 60 μm screen was in use (Fig. 2.5). In the second flow-through tank stocked with 50 larvae/mL (50-B), no overflow occurred during the observation blocks at 60 and 72 hours, when a 41 μm screen was in use. The use of the 41 μm screen (30% OA) continued during the observation blocks at 84 and 96 hours, when overflow of the tank did occur. Banjo screens were fouled from algae clumping in the mesh, suggesting that algae may have been the main cause of clogging of the banjo screen. Once the majority of larvae (>90%) had reached a size greater than 85 μm , the 41 μm banjo screen was exchanged for a 60 μm screen (42% OA). There was no tank overflow during the observation block at 108 hours, when the 60 μm screen was in use (Fig. 2.5).

The catch screens did retain larvae that had been spilled from flow-through tanks due to overflow. When examined, larvae on the catch screens were swimming, but their

guts were lighter in color than larvae that had not been spilled, suggesting those larvae on the catch screen had not been feeding.

Tolerance to metabolic waste

The highest recorded (actual) concentration of ammonia larvae were exposed to in Treatment 1 was 0.4 mg/L, slightly higher than the intended concentration of 0.15 mg/L (Table 2.1). The highest recorded (actual) concentration of nitrite larvae were exposed to in Treatment 1 was 0.017 mg/L, which corresponded with the intended concentration of 0.018 mg/L. The highest recorded (actual) concentration of nitrate larvae were exposed to in Treatment 1 was 9.8 mg/L, higher than the intended concentration of 0.6 mg/L (Table 2.1). Growth of larvae exposed to ammonia, nitrite, and nitrate in Treatment 1 did not differ from growth of larvae in control tanks (Fig. 2.6; Fig. 2.7; Fig. 2.8).

Highest recorded (actual) concentrations of ammonia and nitrite that larvae were exposed to in Treatment 2, 0.25 and 0.036 mg/L, respectively, corresponded with the intended concentrations of 0.3 and 0.036 mg/L, respectively. The highest recorded (actual) concentration of nitrate larvae were exposed to in Treatment 2 was 17.6 mg/L, higher than the intended concentration of 1.2 mg/L (Table 2.1). Growth of larvae exposed to ammonia, nitrite, and nitrate in Treatment 2 did not differ from that of larvae in control tanks (Fig. 2.6; Fig. 2.7; Fig. 2.8).

Highest recorded (actual) concentrations of ammonia and nitrite that larvae were exposed to in Treatment 3, 6.36 and 5.31 mg/L, respectively, were lower than the intended concentrations of 10 and 10 mg/L, respectively. The actual concentration of nitrate larvae were exposed to in Treatment 3 was 26.6 mg/L, higher than the intended concentration of 20 mg/L (Table 2.1). Growth of larvae exposed to nitrite and nitrate in

Treatment 3 did not differ from growth of larvae in control tanks (Fig. 2.7; Fig. 2.8).

Growth of larvae exposed to ammonia in Treatment 3 underwent little change because by day 4 all of the larvae in Treatment 3 were dead (Fig. 2.6).

Ammonia, nitrite, and nitrate were measured in every treatment tank regardless of the metabolic waste being tested to determine if nitrification was occurring. For the ammonia treatments, there was no corresponding increase in nitrite concentrations for any of the three ammonia concentrations tested. Nitrate concentrations were higher than ammonia concentrations on day 4 in Treatment 1 (morning sample: 0.36 mg/L ammonia, 0.44 mg/L nitrate) and on day 4 in Treatment 2 (morning sample: 0.25 mg/L ammonia, 0.35 mg/L nitrate; afternoon sample: 0.2 mg/L ammonia, 0.44 mg/L nitrate).

For nitrite, nitrate concentrations exceeded the nitrite concentrations being tested on day 4 (morning sample: 0.003 mg/L nitrite, 0.27 mg/L nitrate; afternoon sample: 0.002 mg/L nitrite, 0.36 mg/L nitrate) and day 6 (morning sample: 0.005 mg/L nitrite, 0.09 mg/L nitrate; afternoon sample: 0.01 mg/L nitrite, 0.16 mg/L nitrate) in Treatment 1. In Treatment 2, nitrate concentrations exceeded nitrite concentrations on day 4 (morning sample: 0.001 mg/L nitrite, 0.26 mg/L nitrate; afternoon sample: 0.003 mg/L nitrite, 0.4 mg/L nitrate) and day 6 (morning sample: 0.007 mg/L nitrite, 0.04 mg/L nitrate; afternoon sample: 0.01 mg/L nitrite, 0.12 mg/L nitrate). For Treatment 3, nitrate concentrations exceeded nitrite concentrations on every day (Table 2.2).

The highest metabolic waste concentrations tested in Treatment 3 were at least 40 times higher than those observed in the preliminary static system culture, represented by intended concentrations for Treatment 1. The proportion of larvae that survived in Treatment 3 when exposed to nitrite and nitrate was similar to the proportion of larvae

that survived in control tanks, at 1.30 and 0.79 respectively (Fig. 2.9). Survival of larvae in Treatment 3 when exposed to ammonia relative to survival for control tanks was 0, since all larvae were dead by day 4 (Fig. 2.9).

The highest ammonia concentrations recorded in flow-through and static cultures were 0.19 and 0.15 mg/L respectively, lower than the ammonia concentration that caused complete mortality in Treatment 3, which was 6.36 mg/L (Fig. 2.10).

Discussion

Retention of larvae in culture tanks

Overflow of flow-through tanks as a result of clogging of the 41 μm banjo screen did not occur when tanks were stocked with 10 and 20 larvae/mL; therefore, for this thesis system, stocking flow-through cultures with two-day-old larvae at 10 – 20 larvae/mL minimizes the risk of overflow. Flow-through tanks stocked with 50 larvae/mL experienced clogging of the banjo screen and overflow of the tank. The clogging and overflow events were attributed to two main causes: a malfunction in the flow valve used to regulate algae and the use of improper cleaning techniques for the banjo screen (Fig. 2.5).

The persistent overflow in the first tank stocked with 50 larvae/mL (50-A, Fig. 2.5) was probably caused by excess algae present in the tank due to the broken flow valve. The flow valve was not repaired until the observation block at 72 hours, so excess algae (enough to cause a discoloration of the water that was much darker than water of flow-through tanks with functioning flow valves) was present in the tank. The species of algae fed to flow-through cultures were primarily *Pavlova lutherii*, *Chaetoceros* sp. and *Isochrysis galbana*. While all of these species are small enough for larvae to eat, they existed in such excessive numbers within the 50-A tank that larvae were unable to graze down the extra algae, eventually leading to clogging of the banjo screen. Once it had been repaired, the flow valve for algae continued to function correctly for the rest of the

experiment, but the tank continued to overflow as a result of improper cleaning techniques for the banjo screen. For observation blocks at 84 and 96 hours, when the 41 μm screen was in use, the mesh of the banjo screen retained a stained and dirty appearance that persisted despite attempts at cleaning with a fresh water hose. The same stained appearance was noted for the banjo screen in the second tank stocked with 50 larvae/mL (50-B) for observation blocks at 84 and 96 hours (Fig. 2.5). On both banjo screens for the 50 larvae/mL treatments, particles of algae had become embedded within the mesh of the banjo screen, facilitating clogging and overflow. The implementation of a new cleaning procedure for the banjo screen was deemed necessary and is described below. The malfunction of the flow valve for algae was easily remedied through repair, but stressed the importance of regular equipment checks for the flow-through system of this thesis.

The cleaning procedure for the banjo screen that was implemented to prevent clogging and overflow in the flow-through system of this thesis involved rinsing the 41 μm screen with fresh water every 12 hours. Additionally, 41 μm screens were soaked in a mild (approximately 5%) bleach solution for a few minutes every day. After soaking in the bleach solution, banjo screens were rinsed thoroughly with fresh water. When the screen size was increased to 60 μm , the cleaning procedure involved rinsing banjo screens with fresh water once a day, with no soaking in the bleach solution.

Inability to clean the banjo screen thoroughly coupled with the lower percentage of open area in the 41 μm mesh screen (30% OA compared to 42% OA for 60 μm screens), may have facilitated clogging and overflow in the flow-through system of this thesis (Fig. 2.5). Overflow could have been prevented by using the new cleaning

procedure described above or using a mesh screen with a greater percentage of open area to promote water flow. Clogging of the banjo screen and overflow of the tank could also have been avoided by introducing larvae to flow-through culture when they were larger than 85 μm , at approximately four to six days old. At 85 μm , larvae can be retained by a 60 μm banjo screen, which requires less maintenance than the 41 μm banjo screen and has a smaller risk of clogging. Another alternative would be to use a larger banjo screen frame, which would increase the surface area of the mesh screen and reduce the risk of banjo clogging.

Overflow of the tanks should be avoided in the flow-through system because larvae on the catch screens lost the opportunity to feed. Catch screens are not submerged in water, so “spilled” larvae are limited in their feeding and mobility. Larvae can be introduced to this flow-through system at two days old at 10, 20, and 50 larvae/mL provided the proper cleaning procedures for the banjo screen are followed. Subsequent experiments that used this flow-through system implemented the revised cleaning procedures for the banjo screen and observed no overflow events; therefore, experiments using stocking densities as high as 50 larvae/mL were undertaken.

Other studies on flow-through culture have used some form of screen retention, but no mention was made that clogging and overflow occurred (Malouf and Breese, 1977; Southgate and Ito, 1998; Andersen et al., 2000; Magnesen et al., 2006; Sarkis et al., 2006; Rico-Villa et al., 2008; Rico-Villa et al., 2009). The majority of the studies on flow-through culture used a flow rate equivalent to one exchange of water/day (Malouf and Breese, 1977; Southgate and Ito, 1998; Andersen et al., 2000; Magnesen et al., 2006). One study on flow-through culture used a flow rate equivalent to six exchanges of

water/day, for a stocking density of 100 larvae/mL, and did not report any tank overflow (Rico-Villa et al., 2008). Rico-Villa et al. (2008) also used two-day-old larvae, which require a smaller mesh size. A small mesh size, as demonstrated above, can increase the potential for clogging of the banjo screen, yet clogging and overflow were not observed in the Rico-Villa et al. (2008) experiment.

Tolerance to metabolic waste

In Treatment 3 for ammonia, the highest concentration larvae were exposed to was 6.36 mg/L, which resulted in 100% mortality within 48 hours and minimized growth during the short time larvae were alive (Fig. 2.6; 2.9). Ammonia is the most toxic of the three metabolic wastes tested, so these effects were expected. Other studies examining the toxicity of ammonia have reported a wide range of tolerances for different organisms. Among the most tolerant are prawn larvae, *Macrobrachium rosenbergii*. Complete mortality of prawn larvae occurred at 100 mg/L of ammonia at a pH of 8.34 in less than 24 hours (Armstrong et al. 1978). As pH of the seawater becomes more basic, 100% mortality can be induced earlier, since a basic pH increases the amount of ammonia present and consequently its toxicity. The pH that allows for normal growth of oyster larvae is 6.75 – 8.5 (Calabrese and Davis, 1966). In this static system, pH ranged from 6.93 – 8.11.

Timing of exposure in relation to stage of development can influence the susceptibility to ammonia. *Sciaenops ocellatus*, red drum, eggs exposed to 0.55 mg/L of ammonia experienced 100% mortality within the first week of life, whereas three week old red drum experienced only 40% mortality at the same concentration (Holt and Arnold, 1983). For *Penaeus monodon*, tiger prawn, the 24 hour LC₅₀ concentration of

ammonia also increases with age (Chin and Chen, 1987). The same is true for *C. virginica* – larvae in this experiment experienced 100% mortality at 6.36 mg/L, whereas juvenile and adult oysters can tolerate 198 – 1476 mg/L of ammonia (Epifanio and Srna, 1975).

The growth and survival of larvae remained unaffected by nitrite and nitrate. Nitrate is the least toxic of the three metabolic wastes (Epifanio and Srna, 1975), so high tolerance of larvae to nitrate was expected. Larvae also exhibited a high tolerance to nitrite, which is more toxic than nitrate. The major detrimental effect produced by excessive concentrations of nitrite is a decline in pH. A drop in pH below the tolerance range (6.75 – 8.75) of oyster larvae (Calabrese and Davis, 1966) can cause increased mortality, reduced shell growth and calcification, and a reduction in shell thickness (Calabrese and Davis, 1966; Kurihara et al., 2007; Miller et al., 2009). The concentration of nitrite necessary to drop the pH below the tolerance range of larvae is 16 mg/L (Calabrese and Davis, 1966; Collins et al., 1975), greater than the concentration tested in this experiment.

The highest actual concentrations for ammonia and nitrite larvae were exposed to in Treatment 3 were 6.36 and 5.31 mg/L, respectively. The actual concentrations for ammonia and nitrite never met the intended target concentration of 10 mg/L, even though both were replenished to maintain intended concentrations (Table 2.1). Ammonia, nitrite, and nitrate were measured in every experimental tank regardless of the metabolic waste being tested. For nitrite in Treatment 3, there is evidence that nitrification was occurring (Table 2.2). The nitrate concentrations in the tank for Treatment 3 were higher than the recorded concentrations for nitrite, the metabolic waste that was tested. Once oxidized to

nitrate, *P. lutherii*, the species of algae fed to larvae may have assimilated nitrate since nitrogen is a growth limiting nutrient in marine environments (Goldman, 1976).

There is no evidence that ammonia was undergoing nitrification in Treatment 3. Despite the large quantities of ammonia added to the tank, concentrations of nitrite and nitrate showed little corresponding increase. Ammonium chloride, the compound used to test ammonia in this experiment, is soluble in water. According to the material safety data sheet, no chemical reaction will occur when ammonium chloride is dissolved in water, so it is unlikely ammonia was lost through a chemical reaction upon dissolution (HACH Company, 2009). Ammonia can function as a nitrogen source for algae, and can be absorbed by algae without first being oxidized to nitrate by bacteria, so the low concentrations of ammonia present in Treatment 3 may be the result of direct uptake by algae (My Chesapeake, 2011).

Ammonia was monitored in subsequent experiments on flow-through culture in this thesis. It is unlikely that oyster larvae would produce ammonia at a rate that would lead to its accumulation to lethal concentrations (Fig. 2.10), but larvae could still be exposed to high concentrations of ammonia from the outside seawater source. Exchange rates used in this study are fast enough to discourage the accumulation of ammonia in flow-through culture, but will be ineffective at diluting ammonia if concentrations in the seawater source are already at lethal levels; therefore, ammonia concentrations were monitored.

Nitrite and nitrate did not affect development of larvae and both metabolic wastes were tested at levels above concentrations normally observed in static culture. The likelihood that larvae would encounter concentrations of nitrite and nitrate high enough to

cause distress was deemed minimal, so nitrite and nitrate were not measured during subsequent flow-through experiments.

Conclusions

The procedure adopted to eliminate clogging of banjo screens when using a 41 μm mesh was to clean screens every 12 hours and soak them in a mild (5%) bleach solution. The cleaning procedure allowed Eastern oyster larvae to be introduced at two days old at stocking densities as high as 50 larvae/mL in subsequent thesis experiments using flow-through culture. Other procedures that can eliminate clogging of banjo screens and overflow of tanks in this system include delaying introduction of larvae to flow-through culture until they are 85 μm long, increasing the open area of the mesh screen, or increasing the size of the banjo frame to provide a larger surface area for the mesh screen.

Eastern oyster larvae exhibited high tolerance to nitrite and nitrate, but at 6.36 mg/L of ammonia, 100% mortality occurred. The concentrations of ammonia tested exceeded the highest concentrations observed in static and flow-through cultures, so it is unlikely that ammonia concentrations will approach 6.36 mg/L within flow-through cultures for the thesis system. Since ammonia resulted in complete mortality at high concentrations, subsequent thesis experiments using flow-through culture monitored this metabolic waste.

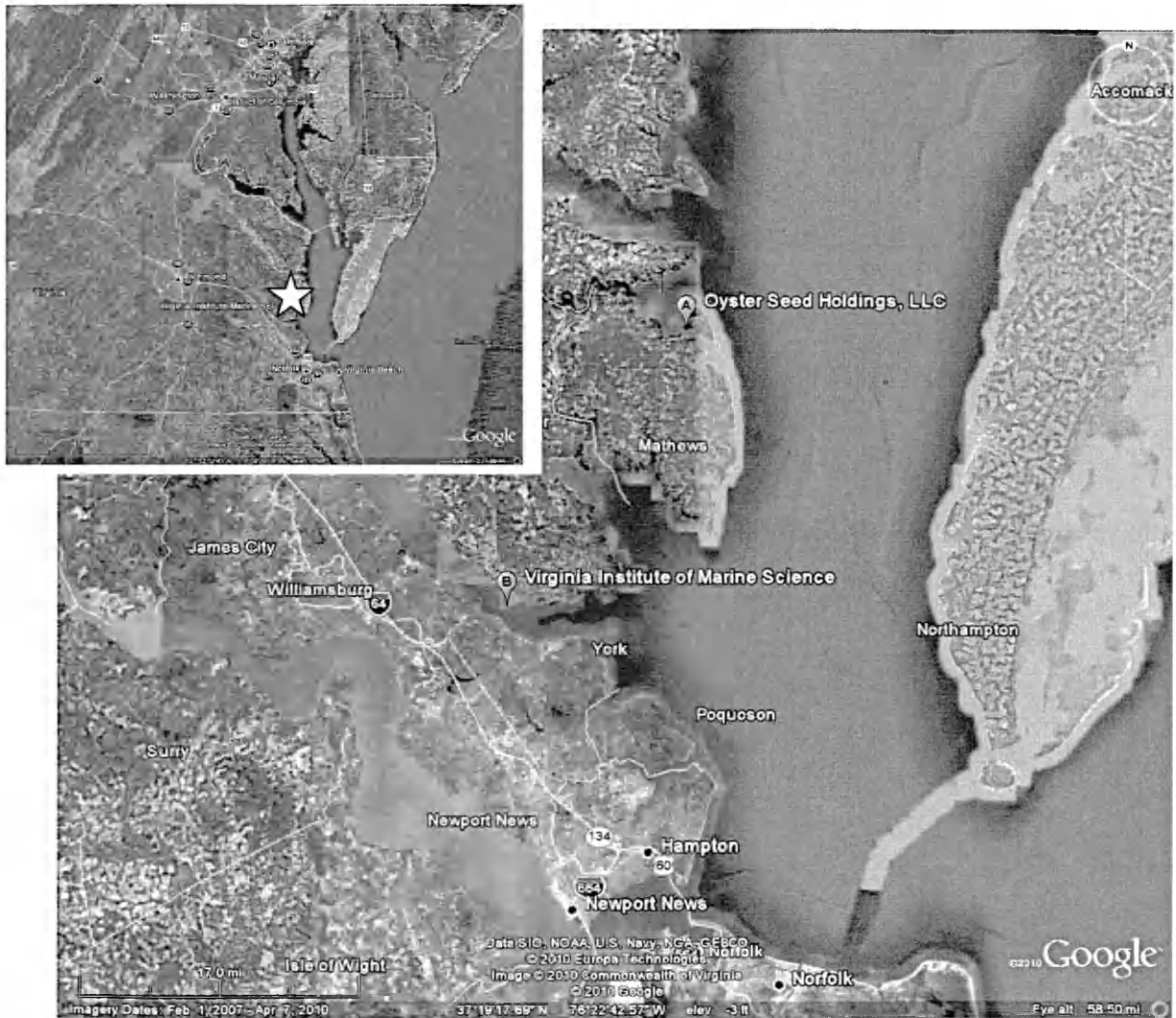


Figure 2.1. The location of Oyster Seed Holdings, LLC on Gwynn's Island (A) and the VIMS hatchery at Gloucester Point (B).



Figure 2.2. Water (top left) and algae (lower right) flow valves for flow-through larval culture.



Figure 2.3. The outflow pipe of one flow-through tank and the Nitex catch screen for “spilled” larvae.

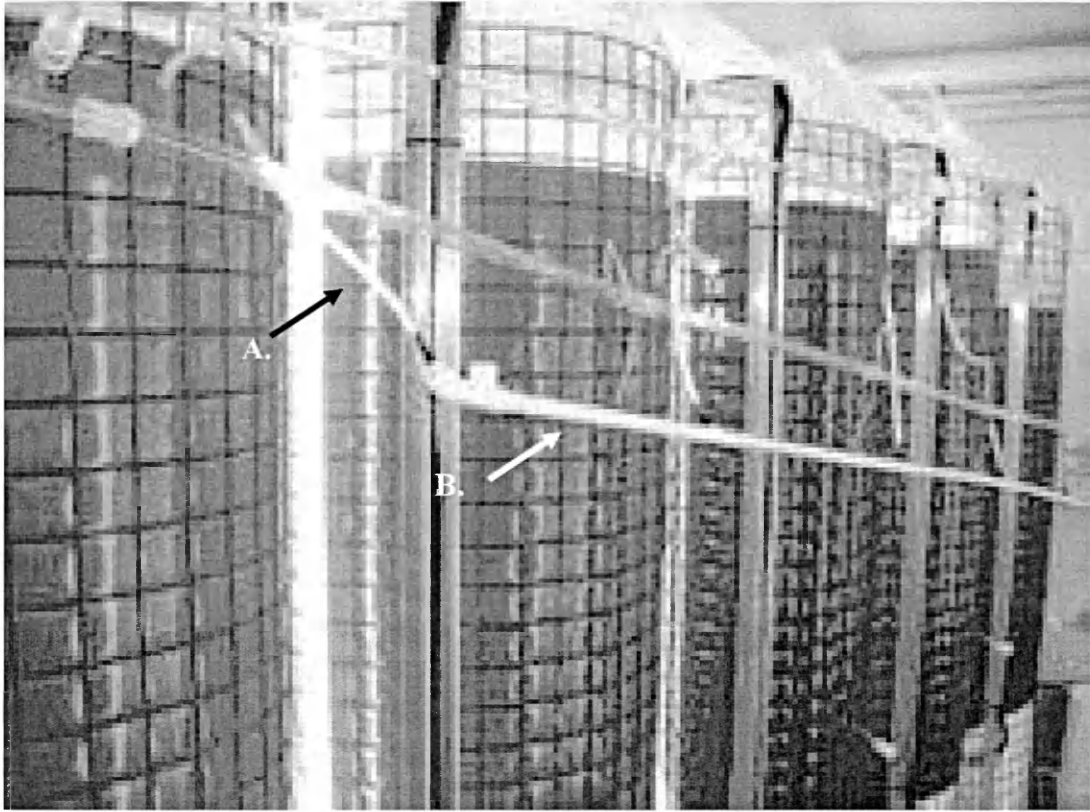


Figure 2.4. The growing system for algae at OSH depicting harvest from each individual growing bag via a connected harvest tube (A) and the pipe that transports the algae to the holding tanks (B).

Table 2.1. The intended concentrations for each metabolic waste (mg/L) tested and the highest concentration actually observed in each treatment. n=1

Nutrient	Treatment 1		Treatment 2		Treatment 3	
	Intended	Actual	Intended	Actual	Intended	Actual
Ammonia	0.15	0.4	0.3	0.25	10	6.36
Nitrite	0.018	0.017	0.036	0.036	10	5.31
Nitrate	0.6	9.8	1.2	17.6	20	26.6

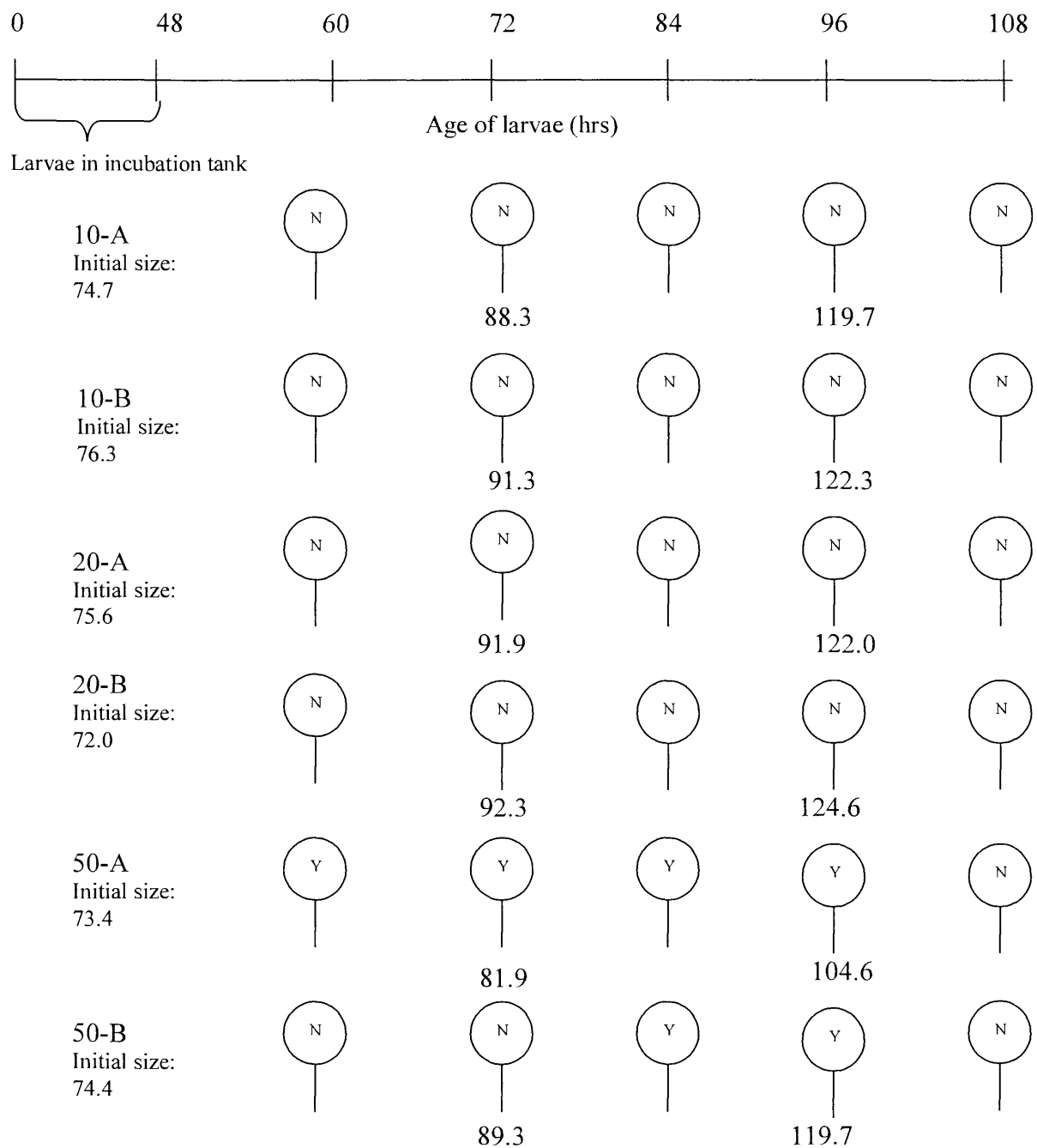


Figure 2.5. The age of larvae for each replicate at 10, 20, and 50 larvae/mL and an indication of whether or not clogging of banjo screens (circle symbols) and overflow occurred. N = no tank overflow occurred, Y = tank overflow occurred. Mean length of larvae (μm) is designated under each banjo screen symbol.

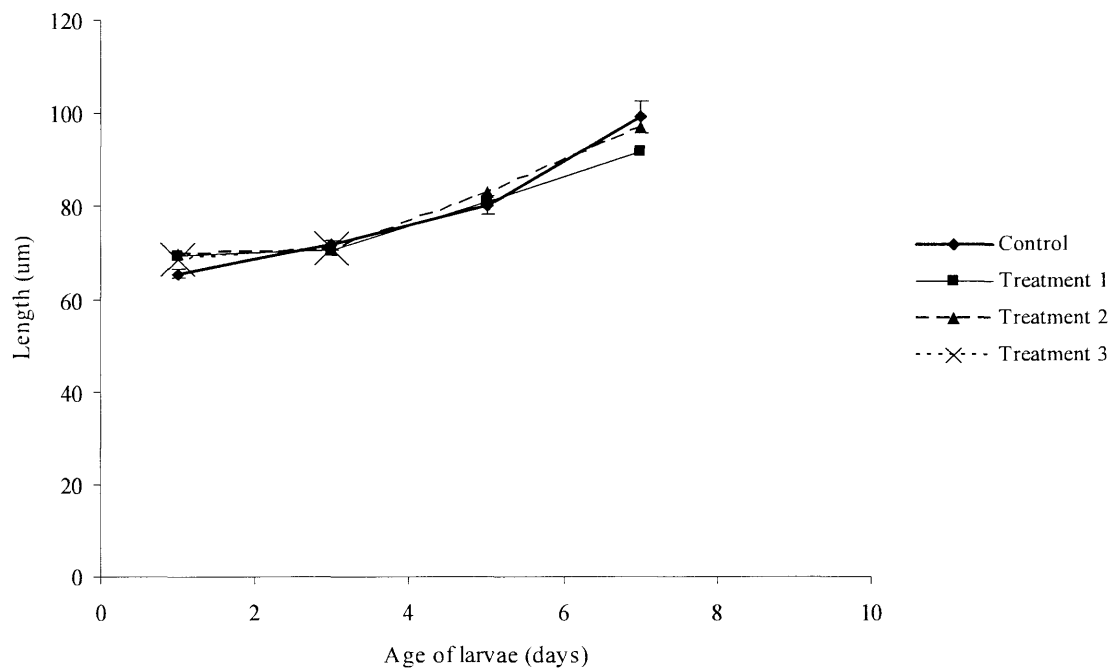


Figure 2.6. The growth of larvae exposed to actual concentrations of 0.4 (Treatment 1), 0.25 (Treatment 2), and 6.36 (Treatment 3) mg/L of ammonia. $n=1$ for metabolic waste treatments, $n=3$ for control.

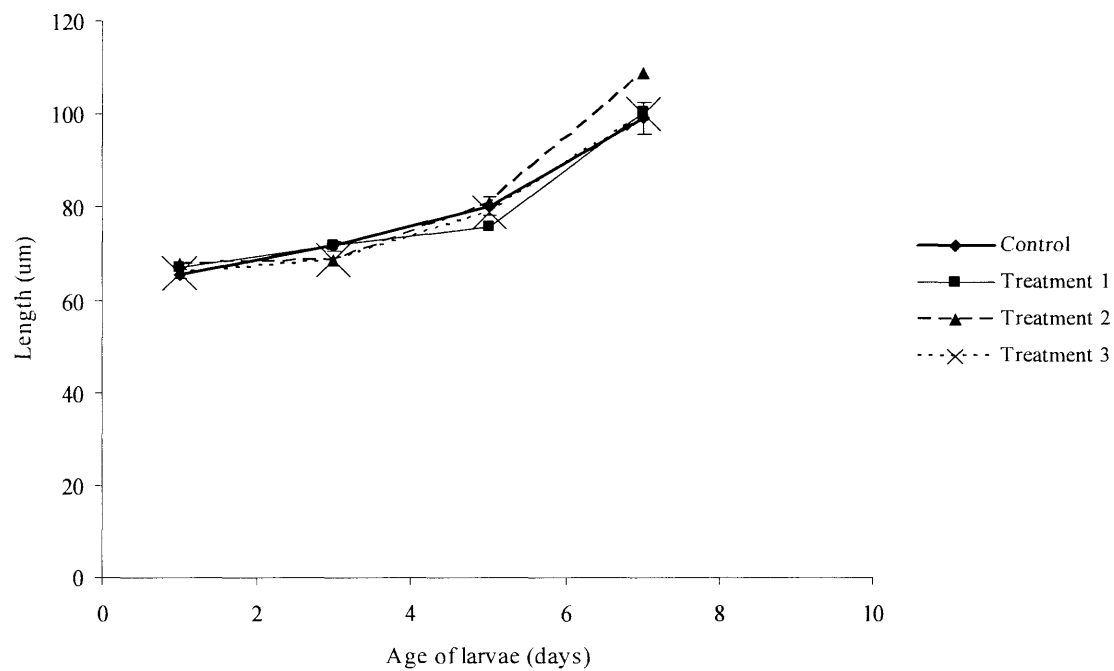


Figure 2.7. The growth of larvae exposed to actual concentrations of 0.017 (Treatment 1), 0.036 (Treatment 2), and 5.31 (Treatment 3) mg/L of nitrite. $n=1$ for metabolic waste treatments, $n=3$ for control.

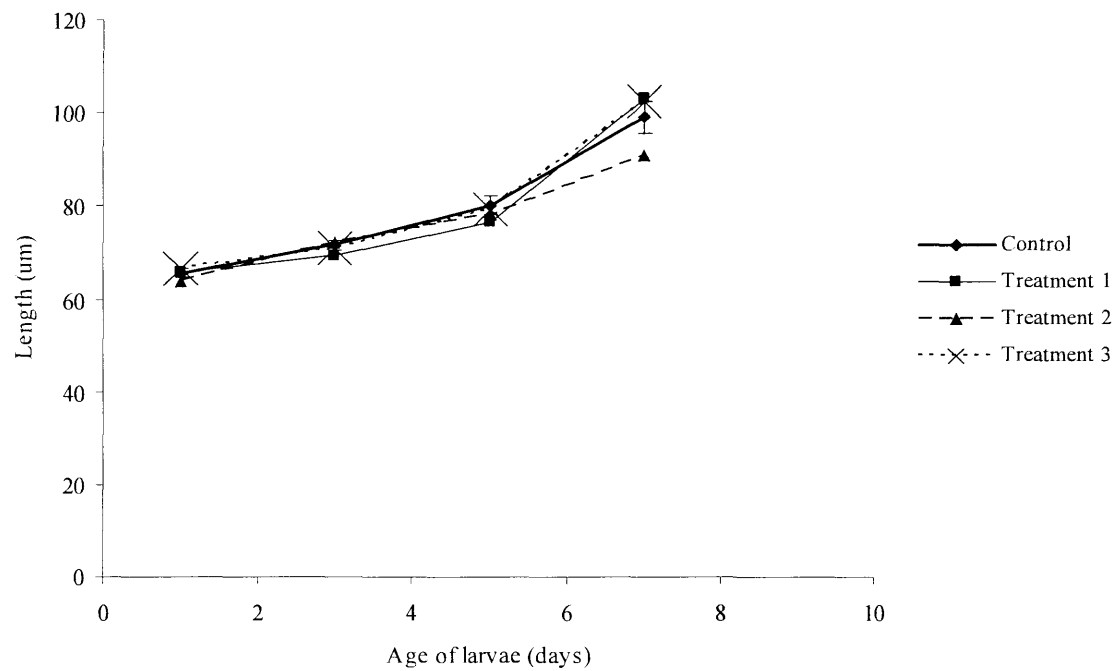


Figure 2.8. The growth of larvae exposed to 9.8 (Treatment 1), 17.6 (Treatment 2), and 26.6 (Treatment 3) mg/L of nitrate. n=1 for metabolic waste treatments, n=3 for control.

Table 2.2. The concentrations of nitrite and nitrate (mg/L) in Treatment 3 when nitrite was tested.

Day	Sampling Time	Nitrite (mg/l)	Nitrate mg/L)
1	morning	1.13	2.4
	afternoon	1.59	2.82
2	morning	3.24	4.41
	afternoon	2.6	4.86
3	morning	2.55	3.52
	afternoon	2.38	3.44
4	morning	3.34	21.9
	afternoon	3.32	34.4
5	morning	2.95	5.2
	afternoon	3.01	4.51
6	morning	3.68	7.95
	afternoon	5.31	8.1
7	morning	4.18	4.84
	afternoon	4.7	4.5

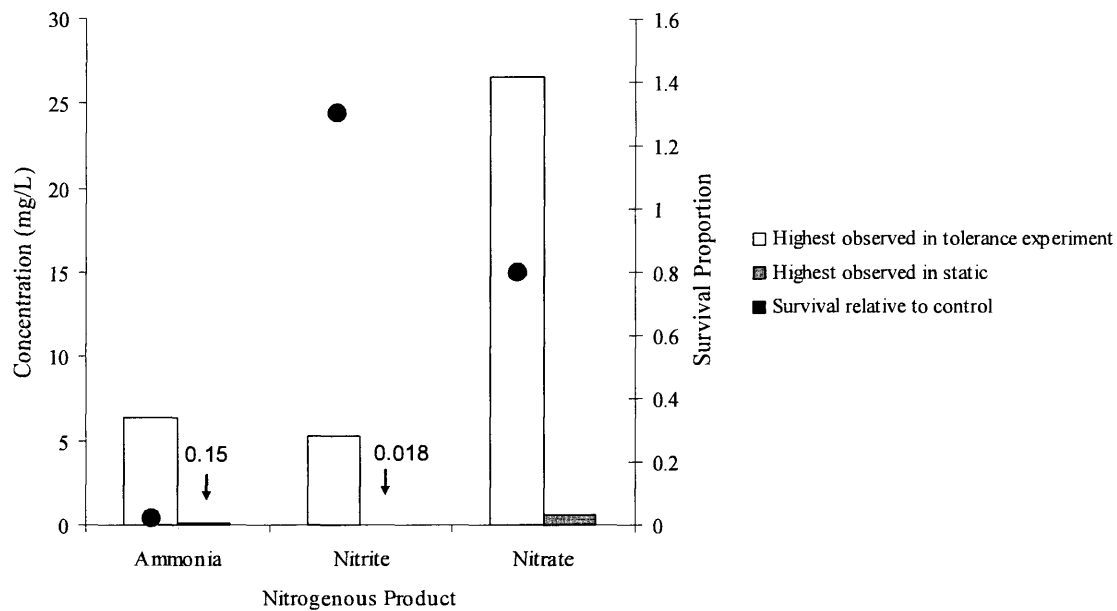


Figure 2.9. The highest metabolic waste concentrations observed in the tolerance experiment (white bar) and the highest metabolic waste concentrations observed in static culture (grey bar). The proportion of survival in each treatment compared to survival of the control (circles) is shown on the secondary y-axis.

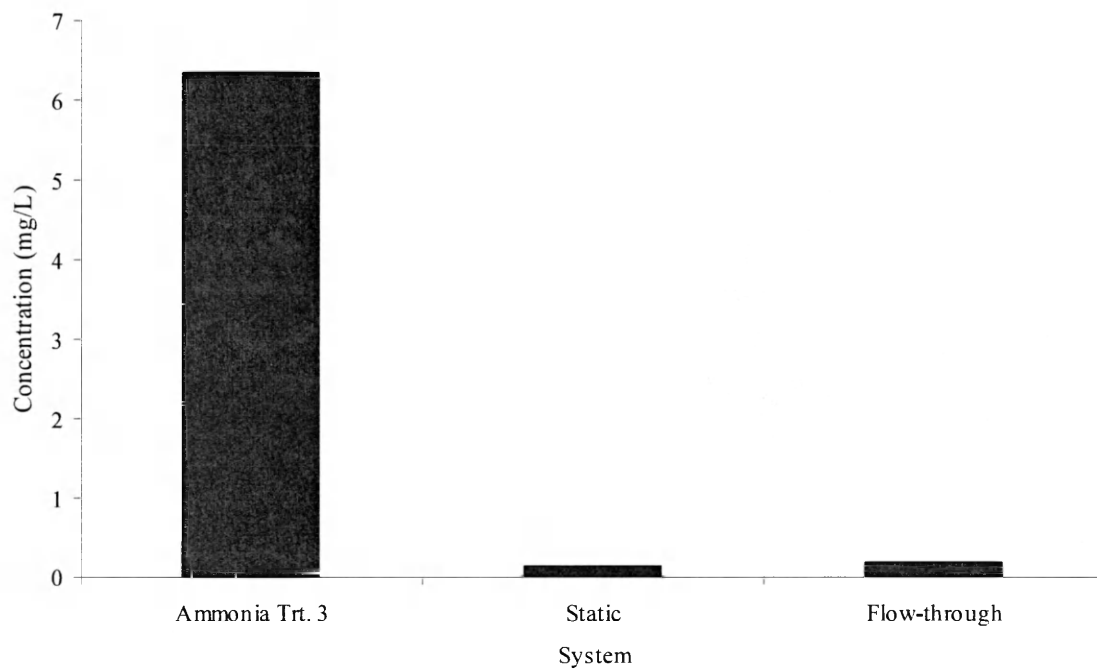


Figure 2.10. The highest ammonia concentrations observed in static culture (for one summer) and flow-through culture (highest out of two summers) compared to the ammonia concentration that resulted in 100% mortality of larvae in the tolerance experiment for metabolic waste.

Chapter Three: The affects of stocking density and exchange rate on survival, growth, competence, and feeding of larvae in flow-through culture

Introduction

Flow-through culture offers the capability of supporting higher stocking densities of larvae than static culture, thereby increasing production of larvae without increasing the space requirements needed for the tank. Increasing stocking density within flow-through culture will only be successful if tank conditions can support the metabolic needs of larvae while preventing the build-up of metabolic waste. Changes in stocking density and flow rate may have unknown affects on the development of larvae. An assessment of the development of Eastern oyster larvae in flow-through culture has not been reported in the literature. There is evidence that larvae of separate bivalve species perform differently when reared in flow-through culture, so results from experiments with one species are not necessarily applicable to another. For example, the development of Great scallop (*Pecten maximus*), calico scallop (*Argopecten gibbus*), and Pacific oyster (*Crassostrea gigas*) larvae differ when these three species are reared in flow-through culture. Great scallop larvae experienced a decline in survival and growth at a stocking density greater than 6 larvae/mL (Magnesen et al., 2006). Calico scallop larvae experienced no difference in survival as stocking density increased from 8 to 24 larvae/mL (Sarkis et al., 2006). Pacific oyster larvae experienced no significant difference in survival, growth, or metamorphic success when reared at high stocking densities of 50 and 100 larvae/mL, yet survival was lower when larvae were reared at 5 larvae/mL (Rico-Villa et al., 2008).

Using the appropriate exchange rate will be critical for obtaining high survival of larvae when stocked at high densities. In two low density studies (ranging from 6 – 24 larvae/mL) conducted by Sarkis et al., (2006) and Magnesen et al. (2006) larvae were reared using exchange rates that ranged from 1 – 3 exchanges of water/day. In one high density study (up to 100 larvae/mL) conducted by Rico-Villa et al. (2008) larvae were reared using six exchanges of water/day, suggesting that as stocking density increases, exchange rate must as well.

The suggestion that as stocking density increases, exchange rate must as well was applied to this thesis study. The development of *Crassostrea virginica* larvae stocked at 10, 20, and 50 larvae/mL in flow-through culture was assessed. Larvae at the three stocking densities were reared at increasing exchange rates: one experiment used five exchanges of water/day, followed by a second experiment that used ten exchanges of water/day. Survival, growth, duration of the competent period, cell consumption, and cell selectivity were examined to determine how they were influenced by stocking density and exchange rate.

Methods

Set-up of the flow-through system

The same flow-through system and sources of brood stock outlined in Chapter 2 were used to test stocking density and exchange rate. Stocking densities of 10, 20, and 50 larvae/mL were each tested under two exchange rates: five (1.4 L/min) and ten (2.8 L/min) exchanges of water/day. The limitation of only having six flow-through tanks made it necessary to conduct the experiments using five and ten exchanges of water/day at separate times during the summer. In both experiments the control was a stocking density of 10 larvae/mL, reared at five exchanges of water/day. A stocking density of 10 larvae/mL was chosen as the control because in a static system stocking density is usually ten D-stage (two-day-old) larvae/mL (Loosanoff and Davis, 1963). An exchange rate of five per day was chosen because it was similar to the exchange rate used by Rico-Villa et al. (2008) in their experiment on high density flow-through culture.

For the experiment that used five exchanges of water/day, 48 female oysters were strip spawned to obtain 180 million eggs. For the experiment that used ten exchanges of water/day, 194 million eggs were obtained from 90 female oysters. All fertilized eggs were held in a 4700 L incubation tank for the first two days of their development, prior to introduction to flow-through culture. Once larvae were 48 hours old, the incubation tank was drained and flow-through tanks stocked at the target densities. Two flow-through tanks were stocked at each density in both experiments: five and ten exchanges of

water/day. Initial stocking densities of 10, 20, and 50 larvae/mL were not maintained during the experiment in order to determine how survival of larvae was affected by the *initial* stocking densities. Future references in this manuscript to treatments of 10, 20 or 50 larvae/mL refers to the initial stocking density in the tank.

Metrics for larvae

Flow-through cultures were drained three times a week and larvae were graded through three or four Nitex mesh screens of decreasing size after each draining. Screen sizes used for grading ranged from 50 – 212 μm and were selected based on the mean length of larvae from the previous drain day. As larvae grew, banjo screens with a larger mesh were used to allow greater water flow. Once each flow-through culture had been drained, larvae were counted to determine survival (using procedures outlined in Chapter Two) and a microscope with a calibrated ocular micrometer was used to measure the length of 35 larvae to obtain mean length of the population. The number of larvae measured was increased from 10 (from Chapter Two) to 35 to provide a more precise estimate for average length.

Harvest of competent larvae began on the first day competent larvae were observed in flow-through culture (denoted by the appearance of a pigmented eye spot). Once harvest of competent larvae began, flow-through cultures were drained every other day until all competent larvae had been harvested. Thirty-five competent larvae were measured for length (in addition to 35 shell lengths for veliger larvae) to obtain mean lengths for competent and veliger larvae. Mean length of competent larvae determined the screen size used to separate them from the rest of the culture. Once competent larvae were separated, they were counted and then refrigerated until a setting assay could be

conducted. For *C. gigas*, refrigeration allows larvae to be held for a week without damage (Budge, 1973). Larvae in the thesis experiment were stored in the refrigerator for no longer than a week; some competent larvae only underwent one day of refrigeration, depending on when they were harvested. A separate count was performed on veliger larvae before they were returned to flow-through culture to determine survival.

The days when 10% and 90% of competent larvae had been harvested from each flow-through culture was calculated. The number of days between and including when 10% and 90% harvest occurred was defined as the duration of the competent period for a given culture.

Feeding

Densities of algae (*Pavlova lutherii*, *Isochrysis* sp., *Chaetoceros* sp., *Tetraselmis suecica*) were monitored using a FLUOROMETER (Turner Designs, Sunnyvale, CA) as outlined in the methods described in Chapter 2. Cell consumption of larvae was calculated by multiplying the number of cells within the flow-through culture (cells/mL) by the flow rate (L/min) and dividing by 0.001 to obtain cells consumed/min. The number of cells consumed/min was multiplied by 60, then by 24 to obtain number of cells consumed/day. The number of cells consumed/day was divided by the number of larvae in the tank to obtain number of cells consumed/larva/day.

The feeding preference of larvae was determined by counting cells based on their volumes (in μm^3) using a Z2 Coulter Counter with Channelizer (Atlanta, GA), a procedure that has been used in several studies on feeding and cell selection of larvae (Malouf and Breese, 1977; Wilson, 1980; Sprung, 1984; Baldwin and Newell, 1995). The Coulter Counter measures number changes in a field across a known aperture. The

aperture has been calibrated to register particles of a known volume, or range of volumes, so counts the number of particles that fall within the specified range. To determine if preferential grazing of algae by larvae occurred, samples were taken from the reservoir and the outflow pipe of each flow-through culture. Using a 1:20 dilution, samples were aspirated through the Coulter Counter to determine the number of cells present within three specified size ranges that distinguished flagellates, diatoms, and *T. suecica*. While *T. suecica* is a flagellate, it is a large cell that larvae are unable to consume until they are older, so *T. suecica* was placed in its own category. The percentage of each cell type present in the reservoir and each outflow sample was calculated, and the difference between them determined (% flagellates in reservoir - % flagellates in outflow of flow-through culture). The difference determined the type of cell that larvae selectively fed on for each day of their development (Malouf and Breese, 1977; Baldwin and Newell, 1995). If the difference was positive, larvae were preferentially consuming that cell type. If the difference was negative, larvae were not preferentially consuming that cell type.

Data for cell consumption was used to calculate growth efficiency of larvae.

Growth efficiency is estimated by the following equation:

$$\text{Growth Efficiency} = \frac{\Delta G}{BA_t} * 100$$

where:

ΔG = change in growth (AFDW) between Time_A - Time_B

BA_t = amount of algae (AFDW) consumed between Time_A and Time_B

To obtain ΔG , the ash free dry weight (AFDW) of larvae was calculated using the following equation (Widdows et al., 1989):

$$AFDW_{\text{larvae}} = (9 \times 10^{-6}) * SL^{2.066}$$

where:

SL = shell length of the larvae (μm)

$AFDW_{\text{larvae}}$ = ash free dry weight of the larvae

The time periods for determining the change in AFDW of the larvae corresponded to the days between filling and draining a tank. Time periods were days 2 – 3, 4 – 5, 6 – 8, etc. The $AFDW_{\text{larvae}}$ was calculated for each time period, meaning that for each time period – days 2 – 3, for 4 – 5, etc – there was an $AFDW_{\text{larvae}}$. The change in $AFDW_{\text{larvae}}$ between two time periods was calculated to obtain ΔG , meaning that the $AFDW_{\text{larvae}}$ for days 2 – 3 was subtracted from the $AFDW_{\text{larvae}}$ from days 4 – 5, etc. Each time period had its own ΔG .

The total biomass of each flow-through culture was calculated by multiplying the number of surviving larvae in the culture by $AFDW_{\text{larvae}}$ for each time period. The total number of cells consumed/larva/day was calculated for each time period and used to obtain the total number of cells consumed/larva/time period.

The AFDW for algae ($AFDW_{\text{algae}}$) was obtained through a literature search of AFDW for the species of algae fed to larvae and averaging those AFDWs, resulting in $7.86 \times 10^{-5} \mu\text{g/cell}$ (Albentosa et al., 1996; Knuckey et al., 2005; Milke et al., 2008). $AFDW_{\text{algae}}$ was multiplied by the total number of cells consumed/larva/time period to obtain the amount of algae consumed within the specified time period (BA_t).

Finally, ΔG and BA_t for each time period were entered into the equation above and multiplied by 100 to calculate growth efficiency.

Setting assay to determine survival to 2 mm

To test the impact of variables on the ability of larvae to undergo metamorphosis and reach approximately 2 mm, Congrove's setting assay for larvae (Congrove, 2008) was performed. The setting assay consisted of three downwellers, all held in a 40 – 60 L plastic bin. Each downweller contained approximately 5 mL microcultch (crushed oyster shell approximately 300 μm in size) in the bottom as a substrate for attachment and metamorphosis of larvae. Tanks and downwellers were cleaned twice a week. For each setting assay, three replicates of 1000 larvae each were isolated from each flow-through culture according to the procedure given below.

All competent larvae from each flow-through culture were pooled in a large beaker of cool water ($\sim 20^{\circ}\text{C}$ to decrease their activity and make them easier to count). A transfer pipette was used to move larvae in small quantities from the beaker of cool water to a Petri dish. Larvae in the Petri dish were counted, and then rinsed into a smaller beaker for holding prior to addition to the downwellers. The transfer procedure was repeated until 1000 individual larvae had been separated from the larger beaker into the smaller one. Each aliquot of 1000 larvae were transferred into the downwellers and allowed to undergo metamorphosis. Larvae that had metamorphosed successfully were kept in downwellers until they could be retained on a 500 μm screen and then transferred to separate upwellers and placed in an outside raceway. When the juvenile oysters could be retained on a 2 mm screen they were counted to determine the number that had survived from the competent stage to 2 mm (Congrove, 2008).

Water quality

A HACH SensIon 156 Portable Multiparameter Meter (Loveland, CO) was used to measure temperature, pH, dissolved oxygen, and salinity. The experiment that tested

tolerance of larvae to metabolic waste (see Chapter Two) indicated that nitrite and nitrate do not have an impact on the survival of larvae. Ammonia is only detrimental to larvae if it occurs in quantities far in excess of what is normally produced by larvae (see Chapter 2). Thus, ammonia was the only metabolic waste monitored. Water samples were tested for ammonia when flow-through cultures were drained. Twice on these days, before draining and after refilling, a 50 mL sample of water from each tank was vacuum-filtered through a 1.5 μm filter disc and stored on ice until it could be processed. A HACH DR/890 Colorimeter (Loveland, CO) was used for ammonia analysis. Procedures for sample analysis are outlined in the HACH Company manual (HACH Company, 2000).

Bacteria levels within each flow-through culture were monitored using TCBS plates. Twice on drain days (before the tank was drained and after it had been refilled) 30 mL of unfiltered water was collected from each tank in a sterilized test tube. The water samples were kept on ice until they could be processed later that day. A dilution that produces between 30 – 100 colony forming units (CFU) per plate is the target density to achieve when growing bacteria (Earle and Crisley, 1975; Nicholls et al., 1976). Diluting the sample to 10^{-1} caused the bacteria count to fall within the designated range. Plates were incubated for 24 hours at room temperature before counting the colonies (Lacoste et al., 2001). Further confirmation on the identity of the bacteria requires immunological and molecular procedures (Harwood et al., 2004), so the counts were used to infer general bacteria loads.

Statistical Analysis

Data are observations on the mean of a population, but due to sampling error and other sources of variability, data can only approximate the true mean of a population.

Experimental means are considered estimates of the real mean of the population. In experiments on flow-through culture, the estimates of means were obtained for survival, growth rate, duration of the competent period, growth efficiency, and cell consumption for larvae reared at five and ten exchanges of water/day. A program in R (ver. 2.8.1), employing general linear model, was used to test the following hypothesis using the models below:

H_O: Survival, growth, duration of the competent period, growth efficiency, and feeding rate of larvae will not be significantly affected by stocking density or exchange rate.

H_A: Survival, growth, duration of the competent period, growth efficiency, and feeding rate of larvae will be significantly affected by stocking density or exchange rate.

The following general linear models use age of larvae, stocking density, and flow-through tank to describe the observed data (survival, growth rate, duration of the competent period, growth efficiency, and cell consumption) while also taking into account interactions that may occur between these parameters (denoted within the parentheses of each model).

Survival

The form of the model is:

$$S_{ijk} = \mu + T_i + D_j + C_k + (T_i * D_j * C_k) + \varepsilon_{ijk}$$

where:

S_{ijk} = survival proportion

μ = survival proportion mean

T_i = age of larvae (days)

D_j = stocking density

C_k = flow-through tank

ε_{ijk} = error

Growth

The growth data for each individual culture was plotted separately and the slope calculated using a linear regression. ANOVA analysis was used to compare differences in growth slopes among treatments. Any data that did not meet the assumptions of normality were log-transformed. The form of the model is as follows:

$$L_{jk} = \mu + D_j + C_k + (D_j * C_k) + \varepsilon_{jk}$$

where:

L_{jk} = slope of growth rate

μ = slope mean

D_j = stocking density

C_k = flow-through tank

ε_{jk} = error

Duration of the competent period

The form of the model is:

$$U_j = \mu + D_j + \varepsilon_j$$

where:

U_j = duration of the competent period

μ = mean for duration of competent larvae

D_j = stocking density

ε_j = error

Growth Efficiency

The form of the model is:

$$GE_{ij} = \mu + T_i + D_j + (T_i * D_j) + \varepsilon_{ij}$$

where:

GE_{ij} = growth efficiency (%)

μ = growth efficiency mean

T_i = age of larvae (days)

D_j = stocking density

ε_{ij} = error

Cell Consumption

The form of the model is:

$$F_{ij} = \mu + T_i + D_j + (T_i * D_j) + \varepsilon_{ij}$$

where:

F_{ij} = # cells consumed/larva/day

μ = # cells consumed/larva/day mean

T_i = age of larvae (days)

D_j = stocking density

ε_{ij} = error

Feeding Selectivity

To determine feeding selectivity, the percentage of each cell type represented in the reservoir and the outflow of each flow-through culture was calculated according to the following:

% cell type X in reservoir - % cell type X in outflow of tank Y = % difference

where:

% difference > 0 indicates larvae are preferentially feeding on that cell type

% difference < 0 indicates larvae are not preferentially feeding on that cell type

Results

Survival to the first day of harvest of competent larvae

At five exchanges of water/day, harvest of competent larvae began on day 11. Mean survival proportion (two replicates) was highest (0.72) to day 11 for the treatment at 20 larvae/mL. Mean survival proportion (two replicates) was lowest (0.29) to day 11 for the treatment at 50 larvae/mL. Mean survival proportion (two replicates) of the control (10 larvae/mL) to day 11 was 0.36. Survival at 20 larvae/mL was significantly higher than 50 larvae/mL (Fig. 3.1; $p < 0.05$). There was no significant difference between the control and 20 larvae/mL treatments or the control and 50 larvae/mL treatments ($p > 0.05$). Larvae numbers declined in all cultures, with final densities at day 11 of 4, 15, and 14 larvae/mL in cultures initiated at 10, 20, and 50 larvae/mL respectively. There was no significant difference in density among treatments when harvest of competent larvae began (Table 3.1; $p > 0.05$). Mean number of competent larvae harvested from each treatment was 1.4 million (10 larvae/mL), 8 million (20 larvae/mL), and 7.6 million (50 larvae/mL) (Table 3.1).

At ten exchanges of water/day, harvest of competent larvae began on day 16. Mean survival proportion (two replicates) was highest (0.55) to day 16 for the control. Mean survival proportion (two replicates) was lowest (0.10) to day 16 for the treatment at 20 larvae/mL. Mean survival proportion (two replicates) at 50 larvae/mL to day 16 was 0.36. There was no significant difference in survival among treatments (Fig. 3.1;

$p>0.05$). Larvae numbers declined in all cultures, with final densities at day 16 of 6, 2, and 16 larvae/mL in cultures initiated at 10, 20, and 50 larvae/mL respectively. There was no significant difference in density among treatments when harvest of competent larvae began (Table 3.2; $p>0.05$). Mean number of competent larvae harvested was 1.8 million (10 larvae/mL), 1 million (20 larvae/mL), and 4.7 million (50 larvae/mL) (Table 3.2).

There was a tank effect that influenced survival of larvae at both exchange rates. Tank 5 was the “best” tank for rearing larvae. At five exchanges of water/day, tank 5 was initially stocked at 20 larvae/mL and had a survival proportion of 0.98 to the first day competent larvae were harvested; at ten exchanges of water/day, tank 5 was initially stocked at 50 larvae/mL and had a survival proportion of 0.66 to the first day competent larvae were harvested (Fig. 3.2). Further investigation into variability among flow-through cultures in the system are reported in Chapter Four.

Survival to the first day competent larvae were harvested from the control (10 larvae/mL) was higher in the experiment that tested ten exchanges of water/day (0.55) than at five exchanges of water/day (0.36). Recall that exchange rate for both controls is five/day. Despite higher survival in ten exchanges of water/day experiment, both controls had a similar total for harvest of competent larvae: 1.4 million at five exchanges of water/day and 1.8 million at the second experiment also reared at five exchanges of water/day (Table 3.1; Table 3.2). Overall survival (from day 2 to last day of harvest) for the controls was 32% (five exchanges of water/day) and 41% (ten exchanges of water/day; reared at five exchanges of water/day) (Table 3.1; Table 3.2). At 20 larvae/mL, survival to the first day competent larvae were harvested was higher at five

exchanges of water/day (72%) than at ten exchanges of water/day (10%). The treatment at 20 larvae/mL reared at five exchanges of water/day had a greater harvest of competent larvae, 8 million, compared to 1 million harvested from ten exchanges of water/day (Table 3.1; Table 3.2). Overall survival (from day 2 to last day of harvest) at 20 larvae/mL was 94% at five exchanges of water/day and 12% at ten exchanges of water/day (Table 3.1; Table 3.2). At 50 larvae/mL, survival to the first day competent larvae were harvested was similar between five and ten exchanges of water/day, 29% and 36%, respectively (Fig. 3.1). Total harvest of competent larvae from treatments at 50 larvae/mL, however, was not similar between the two exchange rates. At five exchanges of water/day, 7.6 million larvae were harvested and at ten exchanges of water/day, 4.7 million were harvested (Table 3.1; Table 3.2). Overall survival (from day 2 to last day of harvest) at 50 larvae/mL was 38% at five exchanges of water/day and 25% at ten exchanges of water/day (Table 3.1; Table 3.2).

Growth, consumption of algae, and feeding selectivity

In the experiment that tested five exchanges of water/day, increasing initial stocking density resulted in slower growth over the course of the larval cycle (Fig. 3.3). At an initial stocking density of 10 larvae/mL (control) veligers grew from a mean of 74 to 242 μm (two replicates). At an initial stocking density of 20 larvae/mL, veligers grew from a mean of 76 to 245 μm (two replicates), the slope of which was not significantly different from the control ($p>0.05$). At 50 larvae/mL, veligers grew from a mean of 75 to 212 μm (two replicates), the slope of which was significantly different from the control ($p<0.05$). Mean lengths (two replicates) for competent larvae harvested on day 11 were 306 μm (10 larvae/mL), 291 μm (20 larvae/mL), and 289 μm (50 larvae/mL) (Fig. 3.3).

For the experiment that tested the more rapid exchange rate of ten/day, stocking density did not significantly affect growth rate of larvae to day 16 (Fig. 3.3; $p>0.05$). In 16 days, veligers in the control grew from a mean of 74 to 210 μm (two replicates), in the treatment at 20 larvae/mL, a mean of 72 to 174 μm (two replicates), and at 50 larvae/mL, a mean of 73 to 155 μm (two replicates). Mean lengths for competent larvae harvested on day 16 were 304 μm (10 larvae/mL), 309 μm (20 larvae/mL) and 297 μm (50 larvae/mL) (Fig. 3.3).

An exchange rate of five/day produced larger veligers on the first day of harvest of competent larvae for all stocking densities. For the controls, ending veliger length was 242 μm at five exchanges of water/day compared to 210 μm in the second experiment also reared at five exchanges of water/day. At 20 larvae/mL, ending veliger length was 245 μm at five exchanges of water/day compared to 174 μm at ten exchanges of water/day. At 50 larvae/mL, ending veliger length was 212 μm at five exchanges of water/day compared to 155 μm at ten exchanges of water/day. Length of competent larvae in the controls was similar – 306 μm at five exchanges of water/day compared to 304 μm in the second experiment also reared at five exchanges of water/day. Treatments at 20 and 50 larvae/mL reared at ten exchanges of water/day had larger competent larvae. At 20 larvae/mL, competent larvae were 291 μm at five exchanges of water/day compared to 309 μm at ten exchanges of water/day. At 50 larvae/mL, competent larvae were 289 μm at five exchanges of water/day compared to 297 μm at ten exchanges of water/day (Fig. 3.3). Treatments that used five exchanges of water/day had a faster growth rate (indicated by slope) compared to ten exchanges of water/day. The slopes for the controls were 0.15 at five exchanges of water/day compared to 0.08 in the second

experiment also reared at five exchanges of water/day. At 20 larvae/mL, slopes were 0.14 at five exchanges of water/day compared to 0.06 at ten exchanges of water/day and at 50 larvae/mL, slopes were 0.12 at five exchanges of water/day compared to 0.06 at ten exchanges of water/day (Fig. 3.3).

The number of cells of algae consumed per larva increased significantly with age for tanks that received five exchanges of water/day (Fig. 3.3; $p < 0.05$). For the control, mean cell consumption (two replicates) ranged from 8,800 – 38,094 cells/larva/day. At 20 larvae/mL, mean consumption (two replicates) ranged from 5,623 – 29,996 cells/larva/day, and at 50 larvae/mL, mean consumption (two replicates) ranged from 3,835 – 32,864 cells/larva/day. There was no significant difference in the number of cells consumed/larva/day among treatments (Fig 3.3; $p > 0.05$).

For the experiment that tested ten exchanges of water/day, cell consumption per larva increased significantly with age (Fig. 3.3; $p < 0.05$). For the control (reared at five exchanges of water/day), mean cell consumption (two replicates) ranged from 21,168 – 143,344 cells/larva/day. At 20 larvae/mL, mean cell consumption (two replicates) ranged from 13,026 – 225,288 cells/larva/day and at 50 larvae/mL, mean cell consumption (two replicates) ranged from 5,750 – 151,118 cells/larva/day. The 50 larvae/mL treatment consumed significantly fewer cells than the 20 larvae/mL treatment ($p < 0.05$). There was no significant difference between the control and 20 and 50 larvae/mL treatments ($p > 0.05$).

Comparing the experiments of five versus ten exchanges of water/day, the control in the second experiment reared at five exchanges of water/day had greater cell consumption (21,168 – 143,344) than the control in the first experiment reared at five

exchanges of water/day (8,800 – 38,094) (Fig. 3.3). At 20 larvae/mL, ten exchanges of water/day had greater cell consumption (13,026 – 225,288) than five exchanges of water/day (5,623 – 29,996) (Fig. 3.3). At 50 larvae/mL, cell consumption was 3,835 – 32,864 at five exchanges of water/day and 5,750 – 151,118 at ten exchanges of water/day (Fig. 3.3).

Growth efficiency is the quantity of biomass produced per unit of food consumed, expressed as a percentage. Greater growth efficiency indicates more biomass was produced with the food consumed. At five exchanges of water/day, mean growth efficiency (two replicates) in the control ranged from 4.9 – 8.7%, at 20 larvae/mL, mean growth efficiency (two replicates) ranged from 12 – 20.3%, and at 50 larvae/mL, mean growth efficiency (two replicates) ranged from 10.3 – 42.7%. There was a significant difference in overall growth efficiency between the control and 50 larvae/mL treatments (Fig. 3.4; $p < 0.05$), but not between the control and 20 larvae/mL or 20 and 50 larvae/mL treatments ($p > 0.05$). On Day 2 there was a significant difference in growth efficiency among the three stocking densities: 8.7% (10 larvae/mL), 18.9% (20 larvae/mL) and 42.7% (50 larvae/mL) (Fig. 3.4; $p < 0.05$).

Mean growth efficiency (two replicates) in the experiment that tested ten exchanges of water/day ranged from 0.7 – 4% (10 larvae/mL), 0.6 – 8.6% (20 larvae/mL), and 3 – 19.5% (50 larvae/mL). There was a significant difference in growth efficiency between the control and 50 larvae/mL and 20 and 50 larvae/mL treatments (Fig. 3.4; $p < 0.05$), but not between the control and 20 larvae/mL treatments ($p > 0.05$). On Day 2 there was a significant difference in growth efficiency among the three stocking densities: 3% (10 larvae/mL), 8.6% (20 larvae/mL) and 19.5% (50 larvae/mL) ($p < 0.05$).

Growth efficiencies were larger for stocking densities reared at five exchanges of water/day than at ten exchanges of water/day. Growth efficiency for controls ranged from 4.9 – 8.7% at five exchanges of water/day and 0.7 – 4% in the second experiment also reared at five exchanges of water/day. At 20 larvae/mL, growth efficiency ranged from 12 – 20.3% at five exchanges of water/day and 0.6 – 8.6% at ten exchanges of water/day and at 50 larvae/mL, 10.3 – 42.7% at five exchanges of water/day and 3 – 19.5% at ten exchanges of water/day.

Feeding preferences were determined by examining three cell types in the mixture that was fed to larvae: flagellates, diatoms, and *T. suecica*. While *T. suecica* is a flagellate, it is a large cell that larvae are unable to consume until they are older, so *T. suecica* was placed in its own category. It should be noted that *T. suecica* comprised less than 1% (by cell count) of the feeding mixture at all times, so larval encounters with this cell type were probably rare.

At five exchanges of water/day, flagellates made up between 18 – 73% of the mixture of algae in the reservoir, diatoms 26 – 82%, and *T. suecica* 0 – 0.01% over the course of the larval life cycle. Most of the time, larvae were feeding primarily on diatoms, as indicated by the change in mean proportion of diatoms (two replicates) between the reservoir and outflow of the experimental tank – ranging from -11 to +64. For flagellates, mean differences (two replicates) were mostly negative – ranging from -60 to +8. Overall, larvae at all three stocking densities were feeding primarily on diatoms even though the majority of the mixture was composed of flagellates (Fig. 3.5).

At ten exchanges of water/day, flagellates made up between 56 – 77% of the mixture in the reservoir, diatoms 22 – 43%, and *T. suecica* 0 – 0.01% over the course of

the larval life cycle. Most of the time, larvae were primarily feeding on diatoms, as indicated by the change in mean proportion (two replicates) of diatoms between the reservoir and outflow of the experimental tank – ranging from -18 to +20. For flagellates, mean differences (two replicates) were mostly negative – ranging from -19 to +19. Overall, larvae were primarily feeding on diatoms even though the majority of the mixture was composed of flagellates (Fig. 3.6).

For both exchange rates, larvae at all stocking densities primarily fed on diatoms even though flagellates were the primary cell type present in the reservoir (Fig. 3.6).

Characteristics of the competent stage

Mean lengths (two replicates) of competent larvae harvested from five exchanges of water/day were 306 μm (10 larvae/mL), 291 μm (20 larvae/mL), and 289 μm (50 larvae/mL). Mean duration of the competent period (two replicates) was four days for both the control (10 larvae/mL) and 20 larvae/mL treatments, and six days at 50 larvae/mL. There was no significant difference in duration of the competent period among these treatments (Fig. 3.7; $p>0.05$). Mean peak harvest (two replicates) (the day when the majority of competent larvae were removed) for the control (10 larvae/mL) and 20 larvae/mL treatments occurred two days earlier than mean peak harvest (two replicates) at 50 larvae/mL (Fig. 3.7). The control and 20 larvae/mL treatments had over 50% of their competent larvae removed at peak harvest, while peak harvest at 50 larvae/mL was 45%. Mean number of competent larvae (two replicates) harvested for each stocking density was 1.4 million (10 larvae/mL), 8 million (20 larvae/mL), and 7.6 million (50 larvae/mL). Although the highest density produced the highest number of competent larvae, overall survival of larvae was lowest (Table 3.1).

Mean lengths of competent larvae (two replicates) harvested from ten exchanges of water/day were 304 μm (10 larvae/mL), 309 μm (20 larvae/mL) and 297 μm (50 larvae/mL). Mean duration of the competent period (two replicates) was seven days for all stocking densities, so there was no significant differences among treatments (Fig. 3.7; $p>0.05$). Mean peak harvest (two replicates) for the control (10 larvae/mL reared at five exchanges of water/day) occurred two days earlier than at 20 and 50 larvae/mL (Fig. 3.7). The control had 34% of competent larvae harvested, while at 20 and 50 larvae/mL, over 50% of competent larvae were removed at peak harvest. The mean number of competent larvae (two replicates) harvested for each stocking density totaled 1.8 million (10 larvae/mL), 1 million (20 larvae/mL), and 4.7 million (50 larvae/mL). Although the highest density produced the highest number of competent larvae, overall survival of larvae was lowest (Table 3.1).

Length of competent larvae in the controls was similar – 306 μm at five exchanges of water/day compared to 304 μm in the second experiment also reared at five exchanges of water/day; however, for treatments at 20 and 50 larvae/mL, larger competent larvae were harvested from ten exchanges of water/day. At 20 larvae/mL, competent larvae were 291 μm at five exchanges of water/day compared to 309 μm at ten exchanges of water/day. At 50 larvae/mL, competent larvae were 289 μm at five exchanges of water/day compared to 297 μm at ten exchanges of water/day (Fig. 3.3). The duration of the competent period was longer for larvae reared at ten exchanges of water/day (Fig. 3.7). For the controls and 20 larvae/mL treatments, duration was four days at five exchanges of water/day compared to seven days at ten exchanges of

water/day. At 50 larvae/mL, duration of the competent period was six days at five exchanges of water/day compared to seven days at ten exchanges of water/day.

Survival to 2 mm

Mean survival proportion (two replicates) from competent stage to 2 mm for five exchanges of water/day was 0.02 (10 larvae/mL), 0.006 (20 larvae/mL), and 0.006 (50 larvae/mL) (Table 3.3). There was no significant difference in survival proportion of competent larvae to 2 mm among stocking densities (Table 3.3; $p>0.05$).

Mean survival proportion (two replicates) from competent stage to 2 mm for ten exchanges of water/day was 0.035 (10 larvae/mL), 0.077 (20 larvae/mL), and 0.017 (50 larvae/mL) (Table 3.4). There was no significant difference in survival proportion of competent larvae to 2 mm among stocking densities (Table 3.4; $p>0.05$).

Survival proportion of competent larvae to 2 mm was greater for larvae reared at ten exchanges of water/day. For the controls, survival proportion was 0.02 at five exchanges of water/day and 0.035 in the second experiment also reared at five exchanges of water/day. At 20 larvae/mL survival proportion was 0.006 at five exchanges of water/day and 0.077 at ten exchanges of water/day. At 50 larvae/mL, survival proportion was 0.006 at five exchanges of water/day and 0.017 at ten exchanges of water/day (Table 3.3; Table 3.4).

Water quality

Temperature, salinity, and pH varied slightly during the course of the hatchery season. For the first half of the season, when the experiment testing five exchanges of water/day was conducted, mean temperature (two replicates) ranged from 24.6 – 27°C (Fig. 3.8), mean salinity (two replicates) ranged from 11.9 – 12.5 (Fig. 3.9), and mean pH

(two replicates) ranged from 7.6 – 8.2 (Fig. 3.10). For the second half of the season, when the experiment testing ten exchanges of water/day was conducted, mean temperature (two replicates) ranged from 24.9 – 29.4°C (Fig. 3.8), mean salinity (two replicates) ranged from 12.6 – 13.7 (Fig. 3.9), and mean pH (two replicates) ranged from 7.6 – 7.8 (Fig. 3.10).

There was no detectable bacteria growth, as indicated by TCBS plates, during the experiment that tested five exchanges of water/day. During the experiment at ten exchanges of water/day, bacteria growth was detected, in single day incidents within a few cultures. The number of bacteria colonies present in the infected tanks ranged from 10 – 660 colonies/mL. The majority of the bacterial infections occurred in tanks stocked at 50 larvae/mL, but only when the water temperature was above 25°C (Table 3.5).

At five exchanges of water/day, ammonia concentrations in all cultures ranged from 0 – 0.18 mg/L and at ten exchanges of water/day from 0 – 0.16 mg/L. There were no discernible patterns either among treatments or over the experimental period. Nor was there a significant difference in ammonia concentrations among treatments or among days during either of the experiments at five or ten exchanges of water/day (Fig. 3.11; $p>0.05$).

Mean ammonia concentrations were calculated for the entire larval period for each stocking density. For the controls, overall mean concentration was 0.09 mg/L at five exchanges of water/day and 0.002 mg/L in the second experiment also reared at five exchanges of water/day). At 20 larvae/mL, overall mean concentration was 0.11 mg/L at five exchanges of water/day and 0.14 mg/L at ten exchanges of water/day, and at 50 larvae/mL, 0.11 mg/L at five exchanges of water/day and 0.02 mg/L at ten exchanges of

water/day.

Discussion

Survival

Survival to the first day of harvest of competent larvae was highest at 20 larvae/mL and five exchanges of water/day. Rearing larvae at ten exchanges of water/day did not improve survival, as treatments stocked at 20 and 50 larvae/mL had survival lower than or equal to the control (Fig. 3.1). At five exchanges of water/day, increasing the stocking density to 50 larvae/mL impaired survival initially, but it should be noted that by the time competent larvae were harvested on day 11, survival was similar between the control and 50 larvae/mL treatments – 36% and 30% respectively. The treatment stocked at 50 larvae/mL and reared at five exchanges of water/day represents the greater loss of larvae (12 million larvae), since it had a larger initial population that experienced heavy mortality during the first 48 hours in flow-through culture (Fig. 3.1; Table 3.1).

Overall, survival to the first day of harvest of competent larvae was low compared to the study by Rico-Villa et al. (2008), who obtained 90% survival at stocking densities of 100 larvae/mL – survival twice as high at twice the stocking density used in the experiment for this thesis. The possibility of rearing Eastern oyster larvae in flow-through culture at high stocking densities should not be discounted based on comparisons with the study performed by Rico-Villa et al. (2008). Though overall survival at 50 larvae/mL and five exchanges of water/day was low (38%), it was not the lowest survival

observed; the lowest was at 20 larvae/mL and ten exchanges of water/day (Table 3.1). There is the possibility that survival of *C. virginica* larvae at high densities in flow-through culture can be improved if a lower exchange rate (below five per day) is used. Comparisons between treatments stocked at 20 and 50 larvae/mL for five and ten exchanges of water/day indicate that survival of larvae between day 2 and first day of harvest of competent larvae declines as exchange rate is increased (Table 3.1; Table 3.2). Further support that a reduction in exchange rate can improve survival is evidenced in the study performed by Sarkis et al. (2006), where there was no difference in survival of larvae as stocking density was increased when three exchanges of water/day (flow rate of 0.48 L/min) was used.

The controls at five and ten exchanges of water/day produced similar amounts of competent larvae: 1.4 million (five exchanges of water/day) and 1.8 million (in the second experiment also reared at five exchanges of water/day). Even though the controls in the first and second experiments were reared at separate times during the summer, overall harvest of competent larvae was similar. Harvest of competent larvae was affected by exchange rate – at five exchanges of water/day harvest of competent larvae was greater compared to harvest at ten exchanges of water/day. Survival from day 2 to last day of harvest of competent larvae was greater at five exchanges of water/day (Table 3.1; Table 3.2).

A tank effect was observed at five and ten exchanges of water/day (Fig. 3.2). Larvae reared in the flow-through tank designated Tank 5 had higher survival at five and ten exchanges of water/day than larvae reared in the other flow-through tanks at both

exchange rates. Further investigation into variation among flow-through tanks and how it affects the variables measured are described in Chapter Four.

Growth, consumption of algae, and feeding selectivity

At five exchanges of water/day, the control (10 larvae/mL) and 20 larvae/mL treatments had the fastest growth rate (Fig. 3.3). At 50 larvae/mL, growth rate was reduced. At ten exchanges of water/day, larvae reared at 20 and 50 larvae/mL failed to grow faster than the control (reared at five exchanges of water/day), indicating that the greater exchange rate failed to improve growth of larvae.

Reduction in growth as a result of increased stocking density has been observed in other studies. Sarkis et al. (2006) and Malouf and Breese (1977) noticed a similar pattern when they reared scallop (*A. gibbus*) and oyster (*C. gigas*) larvae in flow-through culture – increasing stocking density resulted in slower growth rate. The opposite effect was observed by Rico-Villa et al. (2008), who observed that the lowest *C. gigas* stocking density, 5 larvae/mL, had slower growth than the highest stocking density of 100 larvae/mL. Rico-Villa et al. (2008) provide no explanation for this result, and slower growth as a result of low stocking density was not observed in this thesis experiment.

Slower growth experienced by larvae at 50 larvae/mL and five exchanges of water/day is not due to utilization of food, as illustrated by growth efficiency. All stocking densities at five exchanges of water/day consumed virtually the same amount of algae throughout the duration of their culture periods; however, the treatment stocked at 50 larvae/mL had significantly higher growth efficiency than the control, yet still experienced slower growth (Fig. 3.3; Fig. 3.4). The point at which the treatment stocked with 50 larvae/mL slows in growth occurs between days 6 – 9. While all stocking

densities experienced a decline in growth efficiency between days 2 – 4, corresponding to the period of slow growth, the control and 20 larvae/mL treatments almost double their growth efficiency from days 4 – 6, explaining the exponential increase in growth of the larvae observed during this time period. At 50 larvae/mL, a decline in growth efficiency, rather than a doubling, occurs from day 4 – 6, accounting for the growth lag (Fig. 3.3; Fig. 3.4). Acceptable growth efficiency for a marine herbivore is between 10 – 35% (Scott, 1980) and on most days the growth efficiencies of this thesis experiment fell within that range. One speculation for the slower growth experienced at 50 larvae/mL may be the early stress created by stocking flow-through cultures at a high density. The crowded conditions may have stunted larvae at the beginning stages of their development.

At a stocking density of 50 larvae/mL, larvae consumed significantly fewer cells/larva/day than the 20 larvae/mL treatment when both were reared at ten exchanges of water/day (Fig. 3.3). The lower cell consumption did not appear to affect development of larvae at 50 larvae/mL. Not only was growth at 50 larvae/mL similar to the 20 larvae/mL treatment, but peak harvest both treatments occurred on the same day (Fig. 3.7). If lower cell consumption had affected development of larvae at 50 larvae/mL, it should have been reflected in a delay to development to the competent stage.

At ten exchanges of water/day, growth efficiency was significantly different among stocking densities, with 50 larvae/mL having significantly higher growth efficiency than the control and 20 larvae/mL treatments. High growth efficiency at 50 larvae/mL may explain why, despite consuming the fewest cells, its growth rate was similar to the control and 20 larvae/mL treatments (Fig. 3.4). Only on day two did

growth efficiencies in the three stocking densities even approach the expected growth efficiency range for a marine herbivore of 10 – 35% (Scott, 1980). The high cell consumption, most often observed in the control and 20 larvae/mL treatments, may be worth of more investigation and explanation, or may be an anomaly. The data in Chapter Four seeks to establish the cell consumption of oyster larvae when reared in the flow-through system used for this thesis.

Growth efficiency for larvae reared at ten exchanges of water/day was low when compared to growth efficiency at five exchanges of water/day. Larvae reared at ten exchanges of water/day allocated less food to producing biomass than their counterparts at five exchanges of water/day. Slower growth and longer development to the competent stage at 20 and 50 larvae/mL and ten exchanges of water/day is the result of low growth efficiency (Fig. 3.3; Fig. 3.7). There is a significant difference in growth efficiency at day 2 at five and ten exchanges of water/day, which is most likely a product of the growth efficiency calculation (Fig. 3.4). When calculating the change in biomass of a flow-through culture, the biomass of the culture as a whole was used. Biomass of larvae varied based on stocking density – the treatment stocked at 50 larvae/mL had 20 million larvae in the tank initially, so had more biomass than the control, which had 4 million larvae initially. By sheer numbers, the treatment stocked at 50 larvae/mL had the greatest biomass, which would have influenced the growth efficiency calculation. Even though stocking densities were consuming similar amounts of food, the larger numerator (biomass of larvae) favors a larger quotient (growth efficiency).

The number of cells of algae consumed/larva/day increased with age for five and ten exchanges of water/day, which is similar to data obtained by other studies that

examined cell consumption (Rico-Villa et al., 2008; Rico-Villa et al., 2009). Larvae require stored energy reserves to undergo metamorphosis, in addition to maintaining day to day metabolic functions, so the increase in consumption of algae with age is expected (Laing, 1995).

Larvae at all stocking densities for five and ten exchanges of water/day mainly fed on diatoms rather than flagellates or *T. suecica* (Fig. 3.5; Fig. 3.6). Preference for diatoms could be a result of behavioral selection on the part of larvae or a characteristic of diatoms that made them preferable for consumption.

Oyster larvae make little selection among cell types below a size of 10 μm (Fritz et al, 1984). The mechanical inability to differentiate among particles below a certain size means that diatoms and flagellates should have been consumed equally by larvae, since both cells are smaller than 10 μm in size. Preferential consumption of diatoms suggests larvae may not be able to distinguish diatoms from flagellates based on size. Selection could be aided by the differences in physical characteristics between diatoms and flagellates. Diatoms have a silica shell and flagellates do not (Li and Volcani, 1985), so perhaps this trait enabled larvae to distinguish between the two cell types. On some days larvae did preferentially feed on flagellates so this cell type was not entirely ignored (Fig. 3.5; Fig. 3.6).

For *T. suecica*, the negative difference between the reservoir and culture tanks indicates a lack of preferential feeding by larvae. Lack of preferential feeding on *T. suecica* could be because the cell type represented a small proportion of the mixture in the reservoir. Larvae may not have fed on *Tetraselmis* simply because it was scarce. Physiological limitations of larvae could also have influenced their ability to feed on *T.*

suecica. Larvae can consume cells within a size range of 1 – 30 μm , with their preference for larger cells increasing as they grow (Baldwin, 1995). At the beginning of the experiment, the small size of larvae made it difficult for them to feed on *T. suecica*. As larvae grew, some selection for *T. suecica* should have taken place. The fact that there was little selection for *T. suecica* could relate back to its scarcity in the reservoir.

Characteristics of competent larvae

Duration of the competent period lasted between 3 – 7 days at five and ten exchanges of water/day, but the *timing* of harvest varied with stocking density for each exchange rate. At five exchanges of water/day, there was no significant difference in duration of the competent period among stocking densities, yet at 50 larvae/mL, two more days were required for the culture to be entirely harvested and for peak harvest of competent larvae to occur (Fig. 3.7). A similar trend occurred at ten exchanges of water/day. While duration of the competent period was not significantly different among stocking densities, peak harvest in the control (10 larvae/mL reared at five exchanges of water/day) occurred two days earlier than peak harvest at 20 and 50 larvae/mL (Fig. 3.7). The delays observed are most likely due to slower growth, which would have delayed development to the competent stage (Fig. 3.3).

The proportion of competent larvae that metamorphosed and survived to 2 mm showed no discernible trend at five exchanges of water/day (Table 3.3). At ten exchanges of water/day, there was a significant difference for survival to 2 mm. Treatments stocked with 20 and 50 larvae/mL were significantly different from each other, with 20 larvae/mL having the highest survival (Table 3.4).

Survival to 2 mm for larvae reared in the Congrove (2008) experiments ranged from 1 – 80%, with a mean survival of 24%. Survival to 2 mm for all stocking densities at five and ten exchanges of water/day was poor by comparison, ranging from 0.6 – 7.7% (Table 3.3; Table 3.4). The protocol that was followed in this thesis for harvesting competent larvae may have been the cause of low survival. A larva that possessed a pigmented “eye spot” was designated competent and harvested. Competent larvae ranged in size from 288 – 306 μm at five exchanges of water/day and 297 – 309 μm at ten exchanges of water/day (Fig. 3.3). Mean size of competent larvae has been documented between 290 to 310 μm in length (Thompson et al., 1996). Based on the given range for length of competent larvae, harvesting of competent larvae may have occurred too soon in the thesis experiment, as evidenced by the small size. If larvae were not ready to undergo metamorphosis, they may have died in the downweller setting system, explaining the poor survival to 2 mm. A better metric for determining when to harvest competent larvae would have been to take note of the appearance of the crawling foot, in addition to the pigmented eye spot.

A deficiency in diet, in the form of low availability of *T. suecica*, also could be responsible for low survival to 2 mm. One study conducted by Helm (1977) compared growth, metamorphosis, and subsequent development of *O. edulis* larvae fed a mixed diet (*T. suecica* and *I. galbana*) with a single species diet (*T. suecica*). There was no difference in growth between larvae fed the mixed or *T. suecica* diet, but larvae fed the *T. suecica* diet had 45% metamorphic success within the first two days of competence compared with 25% metamorphic success for larvae fed the mixed diet (Helm, 1977). There was no significant difference in size of juvenile oysters between the mixed and *T.*

suecica diets. It is surprising that larvae fed *T. suecica* had similar growth and metamorphic success to larvae fed the mixed diet, because compared to other species of algae commonly used for rearing larvae, the lipid content (a high energy source for larvae) of *T. suecica* is relatively low – 6% for *T. suecica*, 20 – 24% for *P. lutherii* and *I. galbana* and 17 – 19% for *Chaetoceros* sp (Helm et al., 2004). While *T. suecica* cannot meet all the nutritional needs of the larvae, Helm's (1977) data suggest that it is an important component of their diet. Low survival of juvenile oysters to 2 mm at both exchange rates could represent a nutritional deficiency in diet due to lack of *T. suecica*.

Poor survival to 2 mm may have been the result of the downweller setting system. The downweller setting system had been used before, as mentioned earlier, and for that experiment survival to 2 mm ranged from 1 – 80%, with a mean survival of 24% (Congrove, 2008). While the success rate for rearing juvenile oysters to 2 mm in the Congrove (2008) experiment is higher than what was observed at five and ten exchanges of water/day in this thesis experiment, the large range for survival indicates that success from using this downweller system can be highly variable. Additionally, it was observed that some larvae metamorphosed to the sides of the setting box in the thesis experiment, so were unable to contribute to the data for survival to 2 mm.

Due to the difficulty in maintaining both the setting boxes and flow-through cultures at the same time, the setting portion was eliminated for experiments described in Chapter Four.

Water quality

Concentrations of ammonia did not differ significantly with day or stocking density at five and ten exchanges of water/day (Fig. 3.11). Day to day ammonia

concentrations were more likely to be influenced by outside conditions in the river from which seawater was drawn than from flow-through culture conditions in the OSH hatchery. Concentrations of ammonia tended to be higher on days when it rained during this thesis study. Nutrient run-off from the surrounding area can cause an increase in concentrations of ammonia (Paerl, 1997), which could explain the correlation between rainy days and increases in ammonia. Even with the effects of rain, ammonia concentrations never exceeded 0.15 mg/L, so did not approach the concentration necessary to cause severe damage to oyster larvae (see Chapter 2).

Bacteria colonies did not begin to grow on TCBS plates until June, two months after this thesis study began (Table 3.5). For *Vibrio* sp., the number of colonies present in the water column increases with temperature, with propagation beginning at 14°C and stabilizing once temperatures reach 20 – 30°C (Kaneko and Colwell, 1973). In this thesis study, bacteria colonies did not appear until temperatures had climbed above 25°C so were found only during the experiment that tested ten exchanges of water/day (Table 3.5). Once the temperature of seawater was high enough to stimulate growth, bacteria were more likely to grow at the highest stocking density – 50 larvae/mL. The stress on larvae of being stocked at a high density may have resulted in sickly larvae whose presence, coupled with the warm temperatures, encouraged growth of bacteria. Stress can increase the susceptibility of an organism to bacteria, eventually leading to a high mortality rate should the stress persist (Lacoste et al. 2001). At ten exchanges of water/day, the majority of the bacteria colonies did not appear until the later stages of development. As larvae age they become less vulnerable to bacteria infections (Brown, 1973). Competent larvae harvested from flow-through cultures that experienced bacteria

growth were still healthy and able to undergo metamorphosis, though survival to 2 mm was low (Table 3.3; Table 3.4).

Stocking flow-through cultures at low densities (10 – 20 larvae/mL), especially later in the summer when water temperature begins to rise, should discourage growth of bacteria in the flow-through system of this thesis. Bacteria levels are lower in flow-through culture than in static culture, so the threat of bacteria growth is reduced in a flow-through system, if not eliminated (Andersen, 2000).

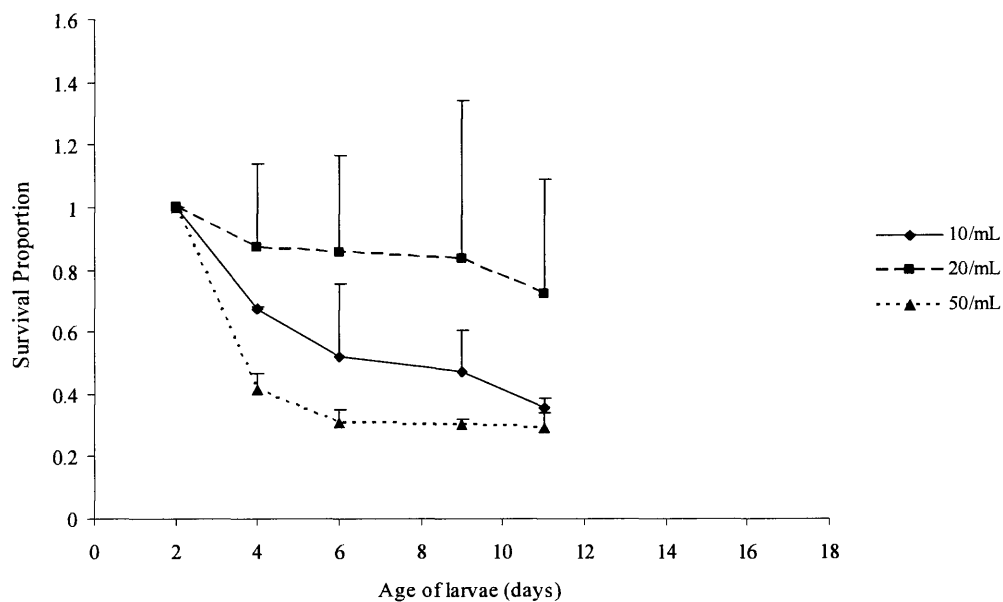
Conclusions

Larvae reared at five exchanges of water/day had higher survival from day 2 to the last day of harvest of competent larvae, faster growth, a shorter duration of the competent period, and a greater harvest of competent larvae at all stocking densities than larvae reared at ten exchanges of water/day in this thesis experiment.

Within the experiment that tested five exchanges of water/day, flow-through cultures stocked at 10 – 20 larvae/mL had higher survival from day 2 to the last day of harvest of competent larvae, faster growth, and a shorter duration of the competent period than flow-through cultures stocked at 50 larvae/mL. The data from this thesis indicate that rearing larvae at 50 larvae/mL within flow-through culture may be a possibility that future studies should examine.

Acceptable levels of ammonia and bacteria colonies were present during the experiments that tested five and ten exchanges of water/day. Concentrations of ammonia were more influenced by conditions in the outside environment than conditions within flow-through cultures. A combination of high water temperature and high stocking density facilitated bacteria growth in the flow-through cultures of this thesis.

A.



B.

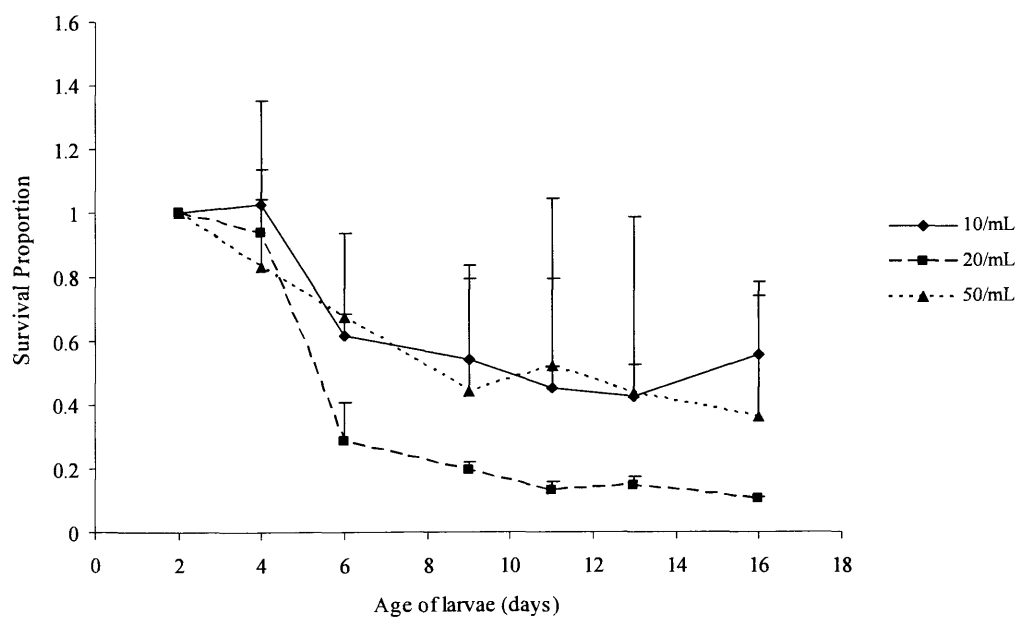


Figure 3.1. Survival proportion of larvae reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Harvesting of competent larvae began at day 11 for larvae reared at five exchanges of water/day and at day 16 for larvae reared at ten exchanges of water/day. Error bars represent +1 sd. n=2

Table 3.1. Stocking density of larvae on day 2 and on first day competent larvae were harvested (day 11) for the replicates that tested five exchanges of water/day. Mean total of competent larvae harvested from each stocking density and mean percent survival of larvae from day 2 to last day competent larvae were harvested from the culture are provided.

Stocking density treatment	Starting density (larvae/mL)	Ending density Rep 1 (larvae/mL)	Ending density Rep 2 (larvae/mL)	Total competent harvested (millions)	Survival % (from day 2 to last harvest day)
Ten larvae/mL	10	3.4	4.1	1.4	32
Twenty larvae/mL	20	10.1	20.7	8	94
Fifty larvae/mL	50	15.1	13.6	7.6	38

Table 3.2. Stocking density of larvae on day 2 and on first day competent larvae were harvested (day 20 for rep 1 of 50/mL treatment, day 16 for all other treatments) for the replicates that tested ten exchanges of water/day. Mean total of competent larvae harvested from each stocking density and mean percent survival of larvae from day 2 to last day competent larvae were harvested from the culture are provided.

Stocking density treatment	Starting density (larvae/mL)	Ending density Rep 1 (larvae/mL)	Ending density Rep 2 (larvae/mL)	Total competent harvested (millions)	Survival % (from day 2 to last harvest day)
Ten larvae/mL	10	6.9	5.3	1.8	41
Twenty larvae/mL	20	2.4	2.1	1	12
Fifty larvae/mL	50	0.5	31.5	4.7	25

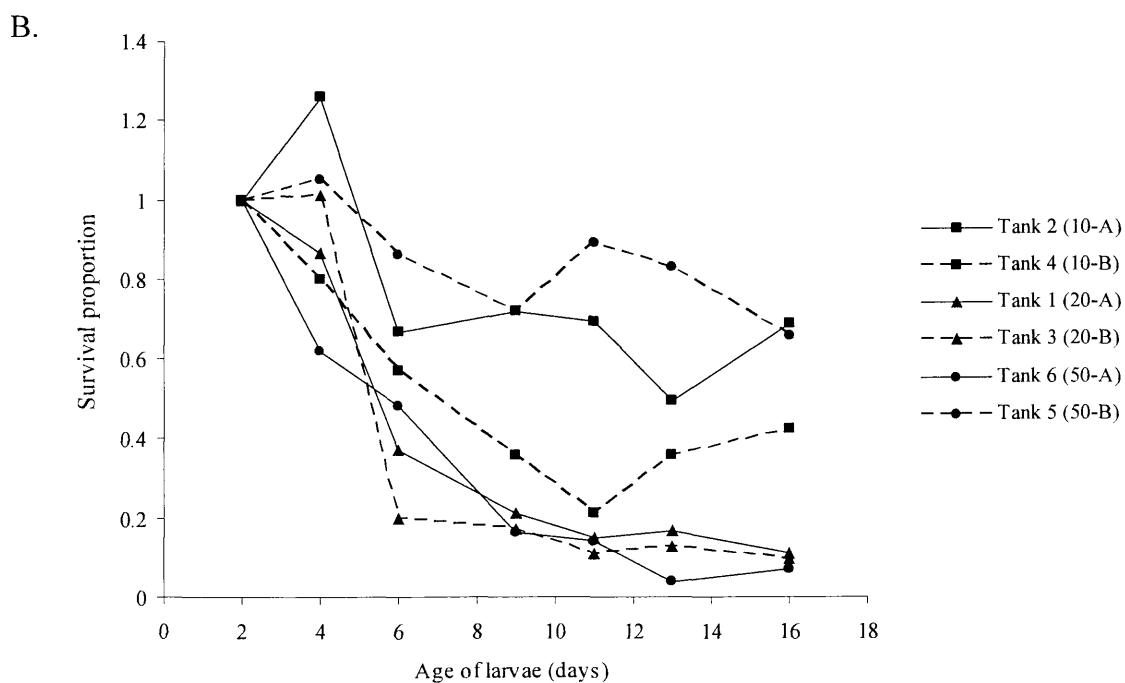
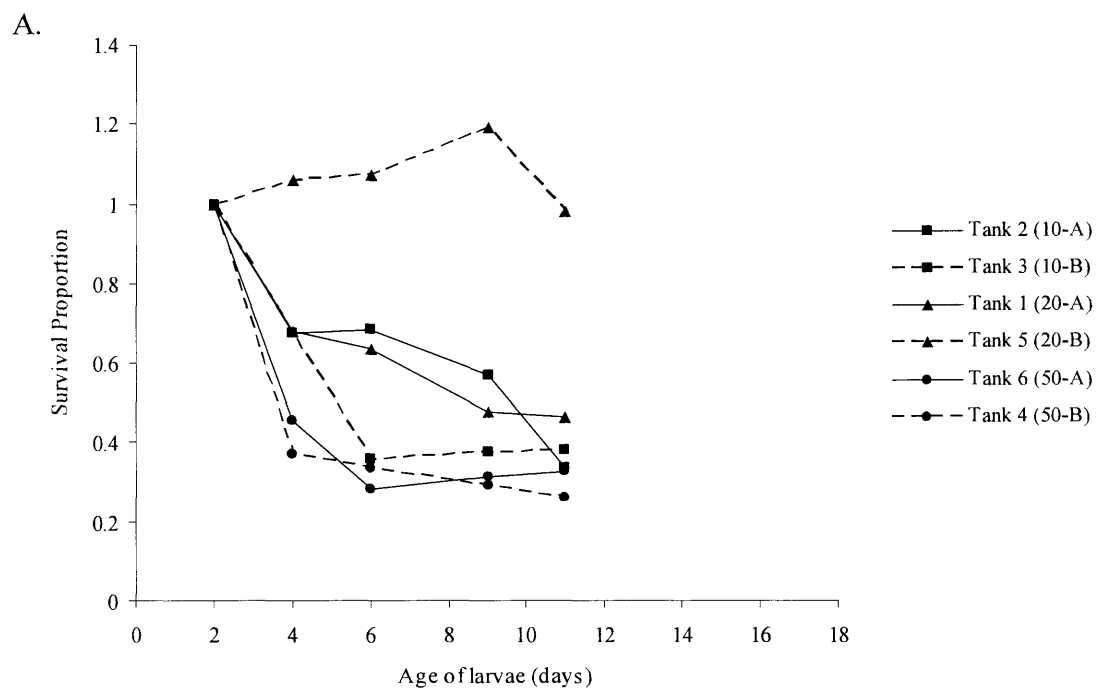


Figure 3.2. Survival proportion of larvae reared at five exchanges of water/day (A) and ten exchanges of water/day (B) to illustrate the affect of Tank 5 on survival of larvae.

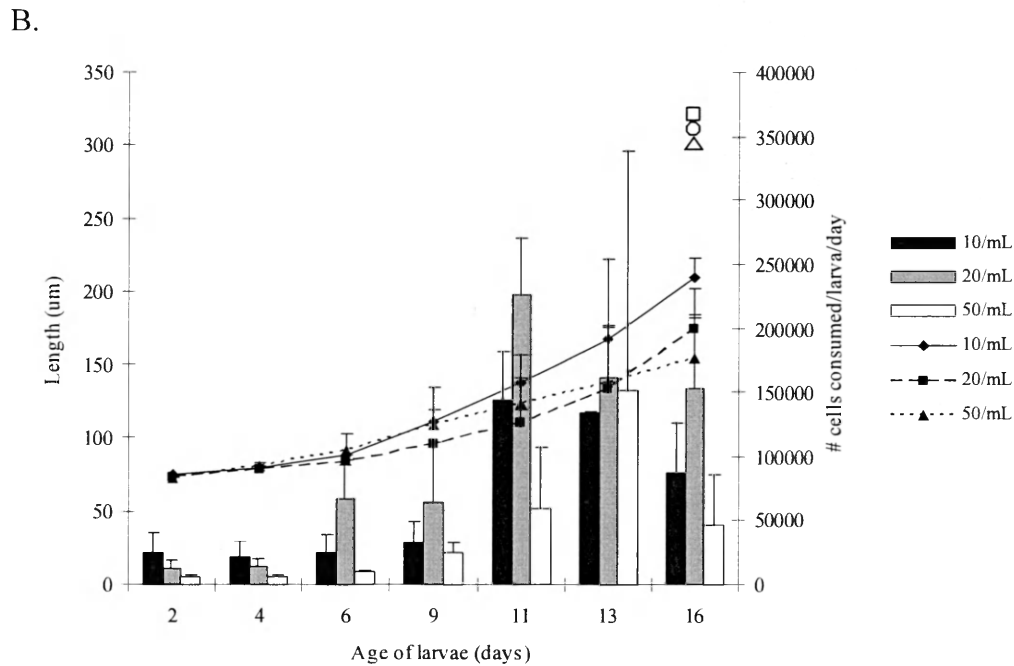
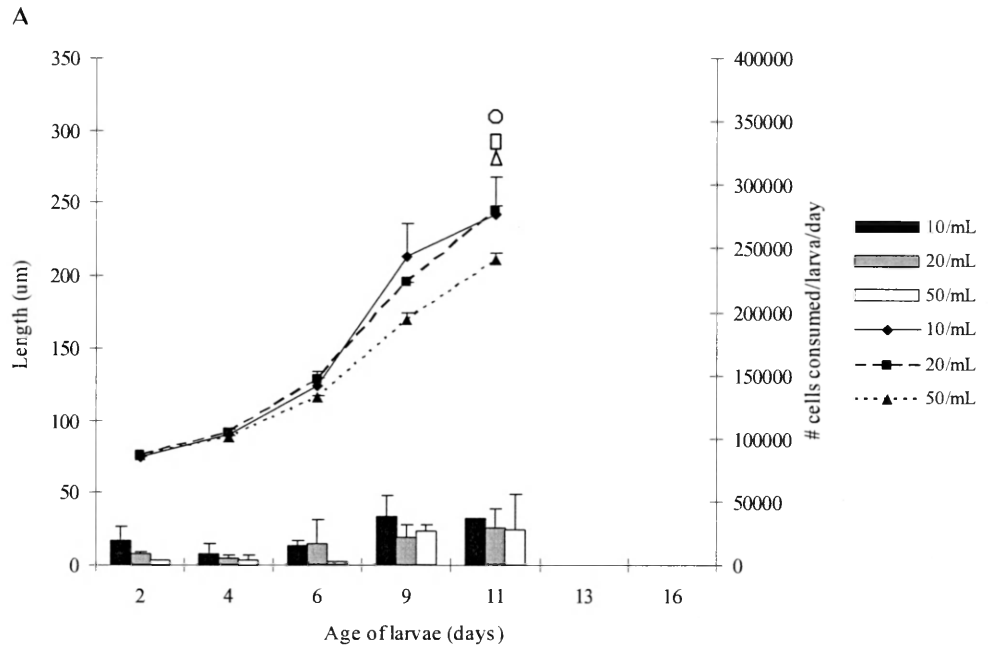


Figure 3.3. Length of veliger larvae (lines) and number of cells consumed/larva/day (bars) at five exchanges of water/day (A) and ten exchanges of water/day (B). Harvesting of competent larvae began on day 11 for five exchanges of water/day and day 16 for ten exchanges of water/day. Separate points on day 11 (A) and day 16 (B) represent mean length of competent larvae for 10 larvae/mL (circle), 20 larvae/mL (square) and 50 larvae/mL (triangle). Error bars represent +1 sd. n=2.

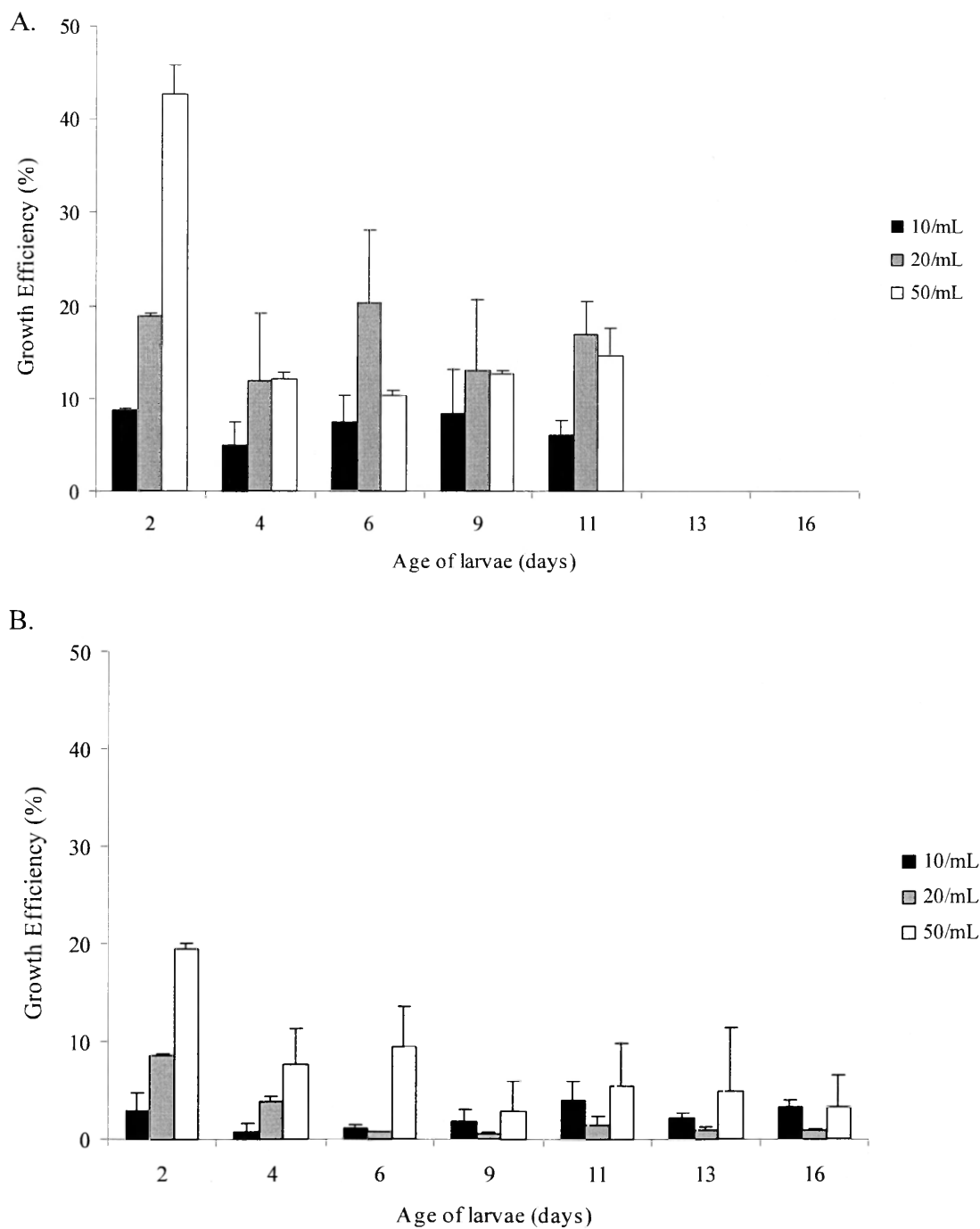


Figure 3.4. Growth efficiency of larvae stocked at 10, 20, and 50 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars = +1 sd. n=2

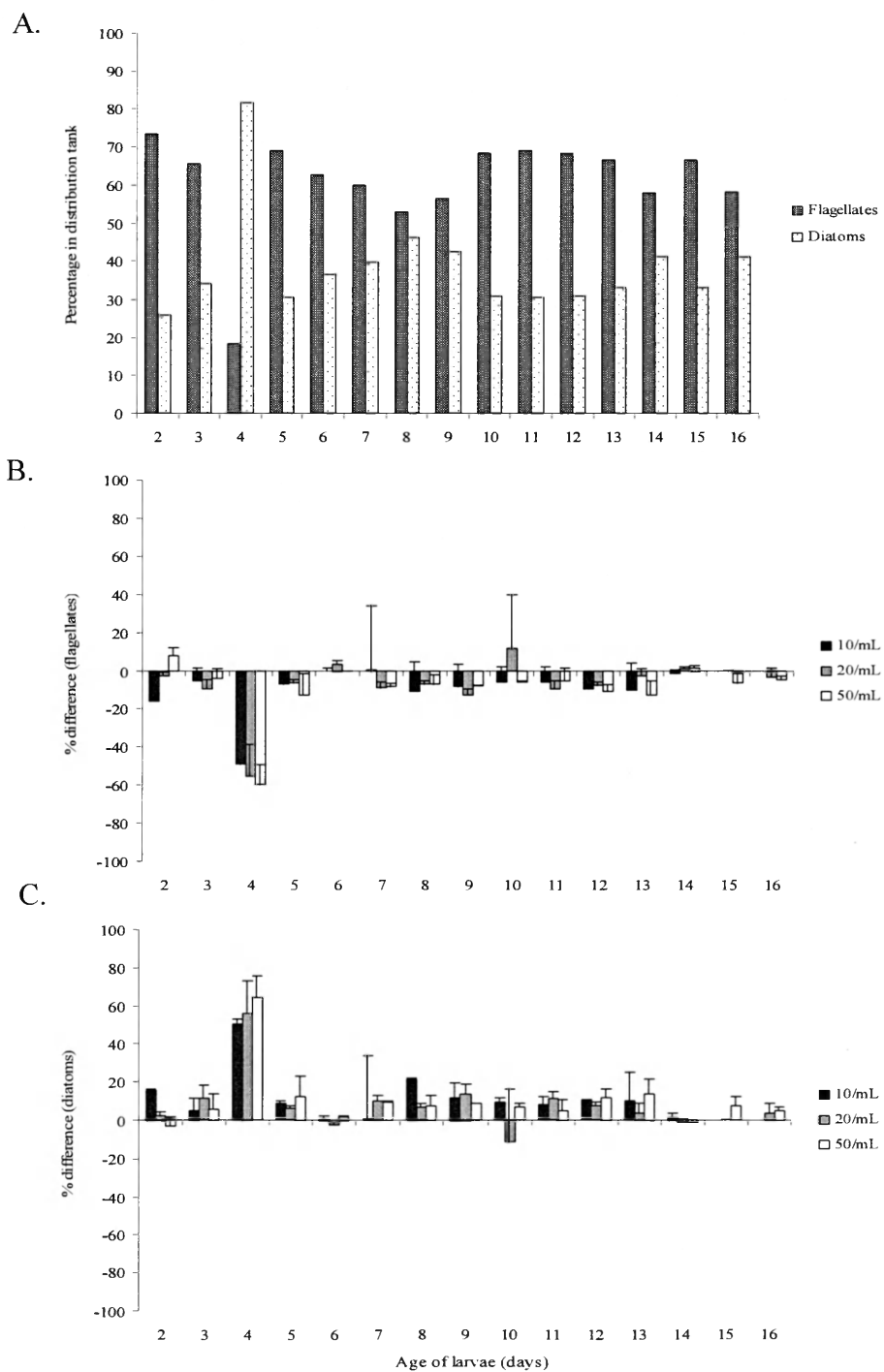


Figure 3.5. Percentage of flagellates and diatoms in the reservoir during development of larvae reared at five exchanges of water/day (A). Percent difference between the reservoir and outflow of flow-through culture for flagellates (B) and diatoms (C). Error bars = +1 sd. n=2.

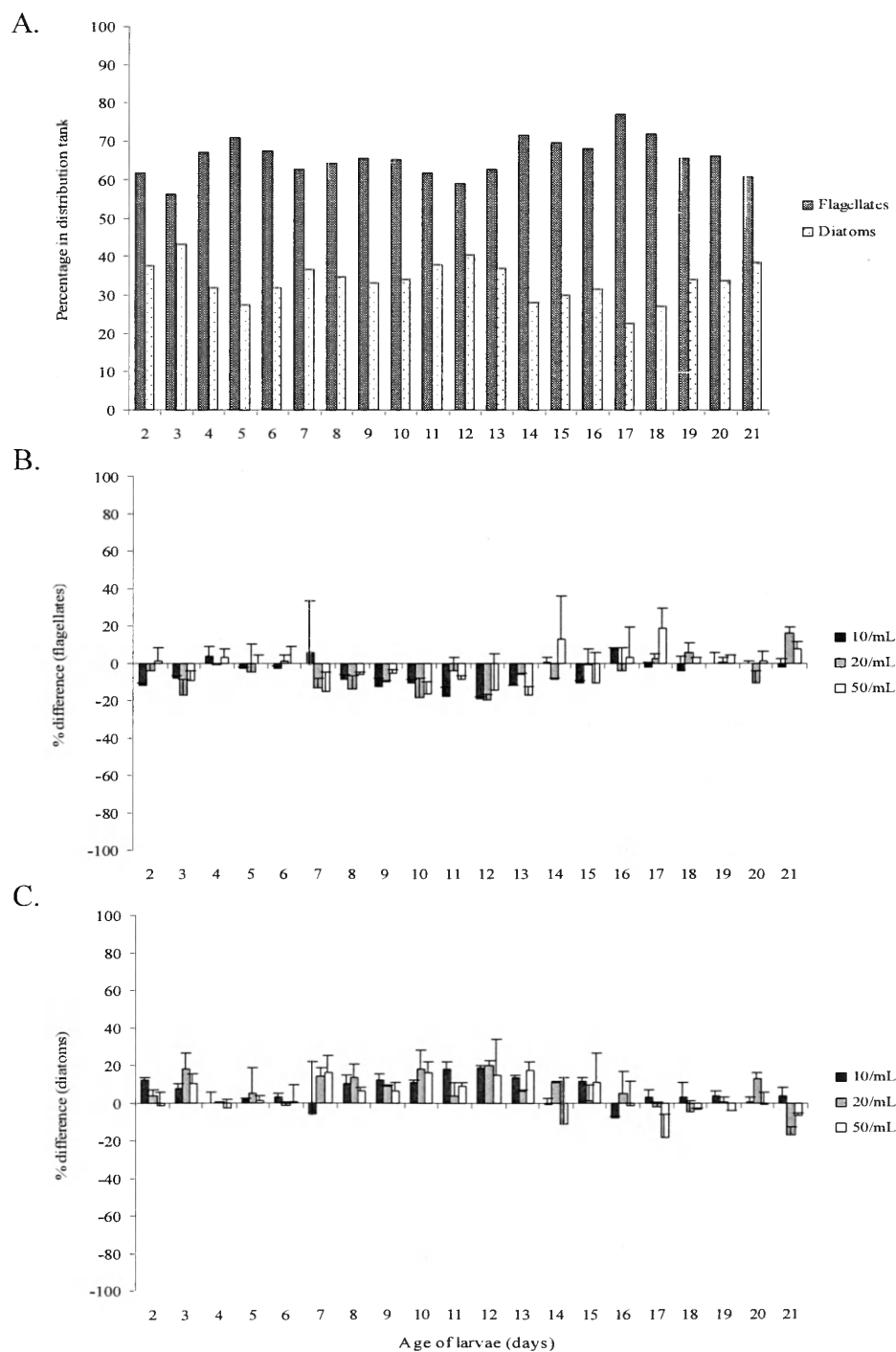
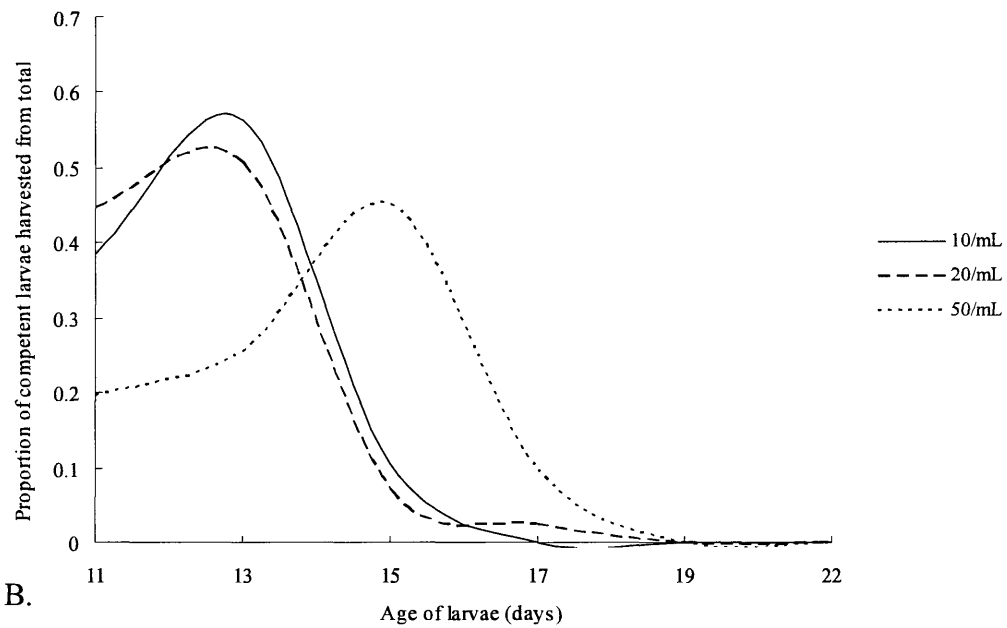


Figure 3.6. Percentage of flagellates and diatoms in the reservoir during development of larvae reared at ten exchanges of water/day (A). Percent difference between the reservoir and outflow of flow-through culture for flagellates (B) and diatoms (C). Error bars = +1 sd. n=2.

A.



B.

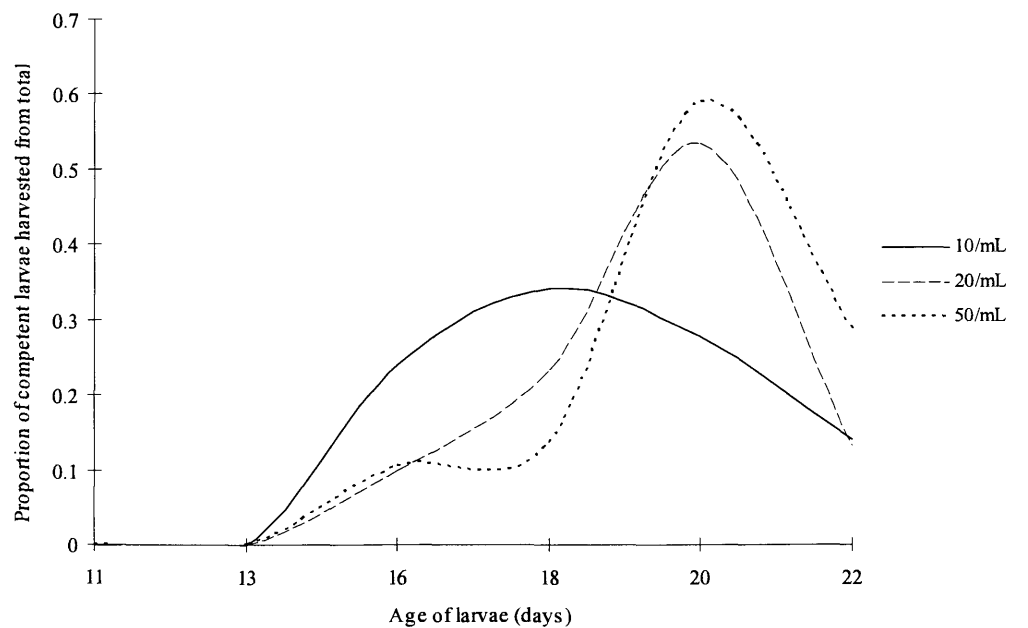


Figure 3.7. Proportion of competent larvae (from total number harvested from each stocking density) removed on each drain day from flow-through cultures reared at five exchanges of water/day (A) and ten exchanges of water/day (B).

Table 3.3. Mean survival proportion to 2 mm for flow-through cultures stocked at 10, 20, and 50 larvae/mL and reared at five exchanges of water/day. n=6

Stocking Density (larvae/mL)	Average Competent		Survival Proportion
	Larvae Set	Average to 2 mm	
10	999.5	19.3	0.020
20	1044	6.2	0.006
50	1017	5.8	0.006

Table 3.4. Mean survival proportion to 2 mm for flow-through cultures stocked at 10, 20, and 50 larvae/mL and reared at ten exchanges of water/day. n=6

Stocking Density (larvae/mL)	Average Competent Larvae		Survival Proportion
	Set	Average to 2 mm	
10	1034	35.8	0.035
20	1021	78.5	0.077
50	1010	16.8	0.017

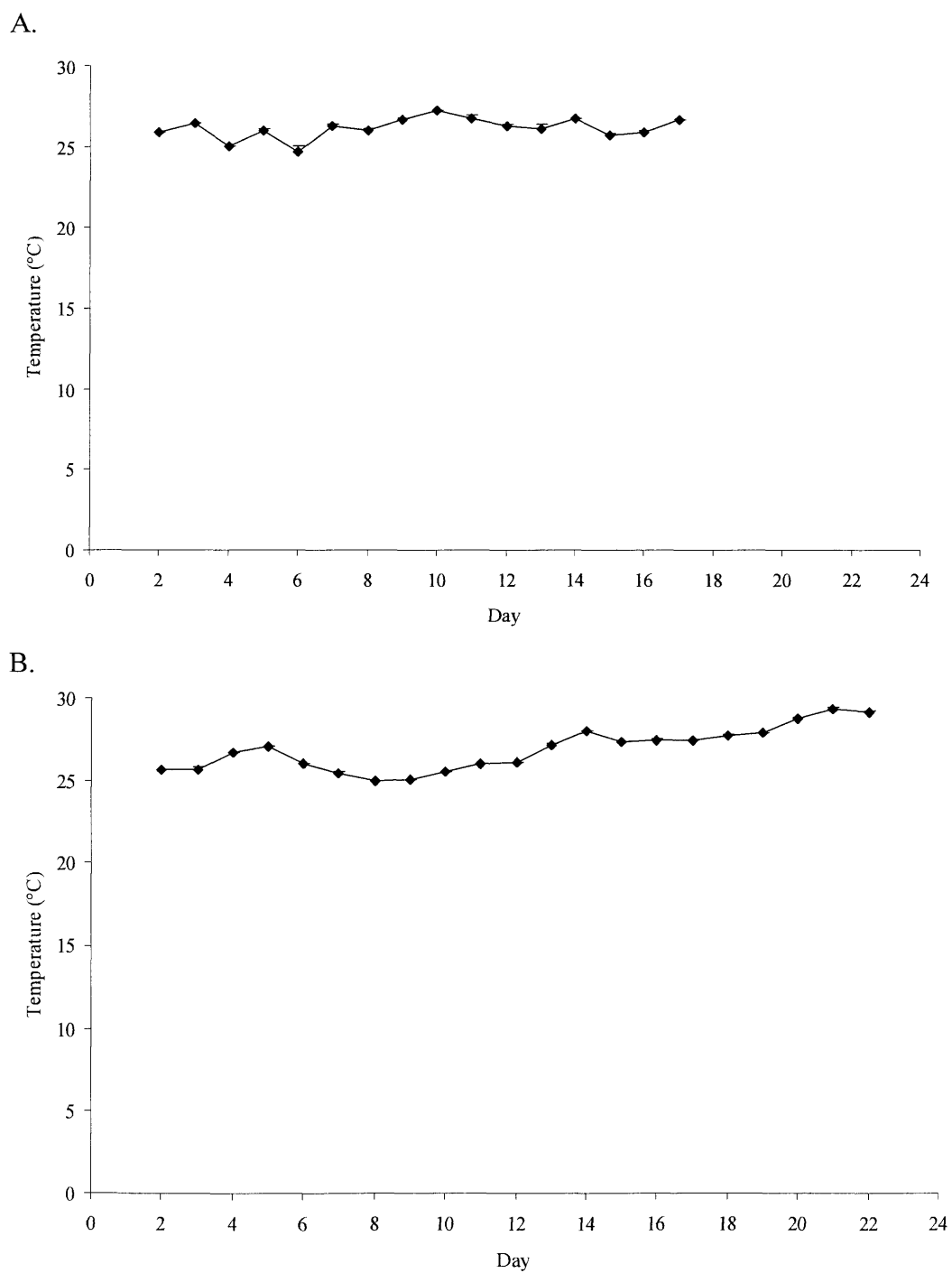
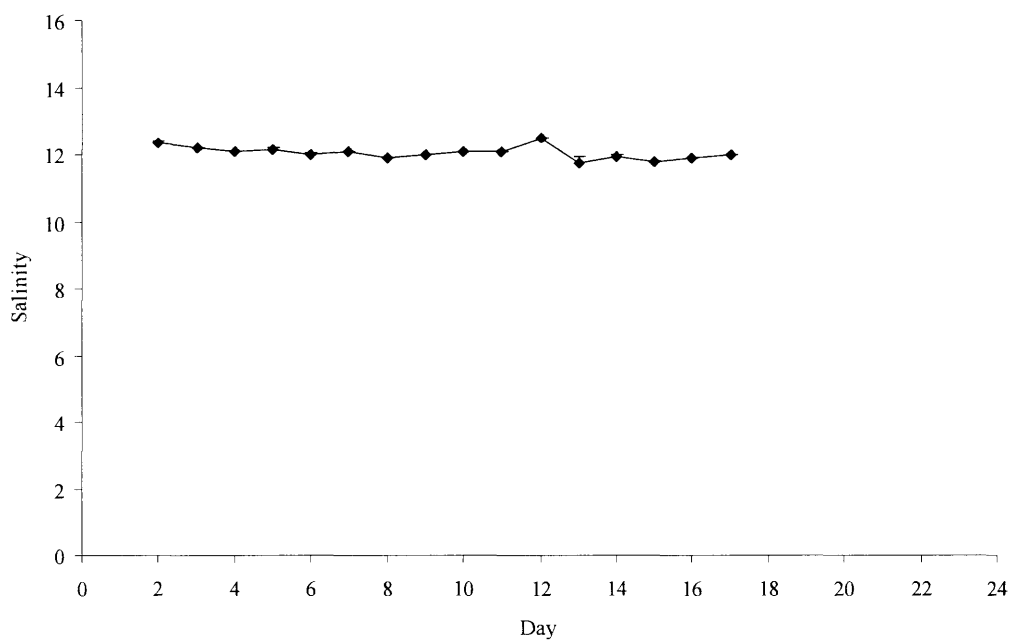


Figure 3.8. Mean daily temperature for flow-through cultures at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. n=6.

A.



B.

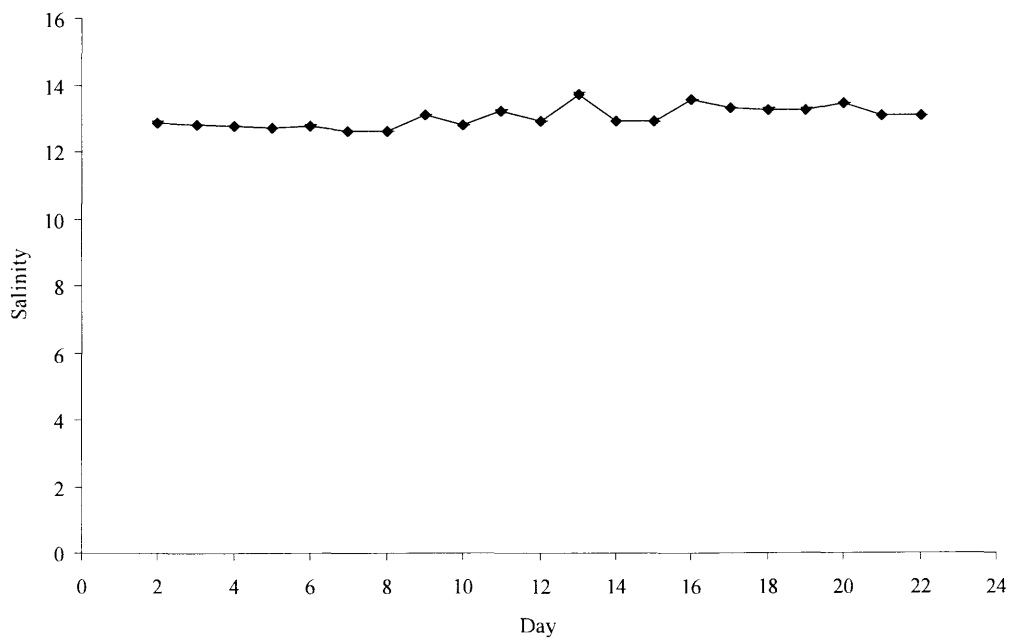


Figure 3.9. Mean daily salinity for flow-through cultures at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. n=6.

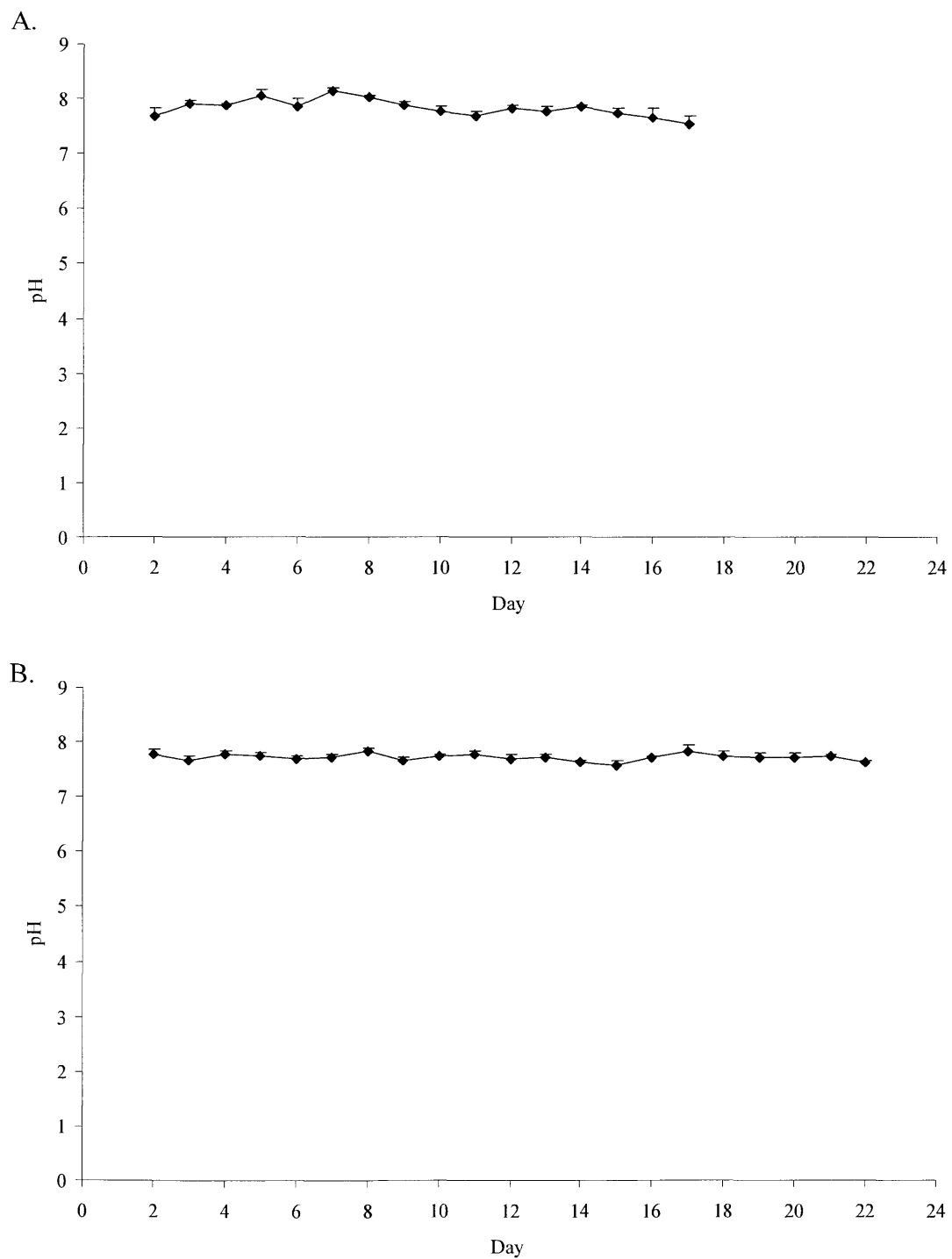


Figure 3.10. Mean daily pH for flow-through cultures at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent ± 1 sd. $n=6$.

Table 3.5. Days and stocking densities that had bacteria growth on TCBS plates reared at ten exchanges of water/day. No bacteria were seen in the experiment that tested five exchanges of water/day.

Date	Day	Water Temp (°C)	Treatment	Tank	No. colonies/mL
6/3/2010	2	25.6	50/mL, rep 1	6	110
6/17/2010	16	27.4	10/mL, rep 2	4	20
6/21/2010	20	29.1	50/mL, rep 2	5	10
6/21/2010	20	28.6	50/mL, rep 1	6	650
6/23/2010	22	29.2	50/mL, rep 1	6	40
6/23/2010	22	29.2	20/mL, rep 1	1	660

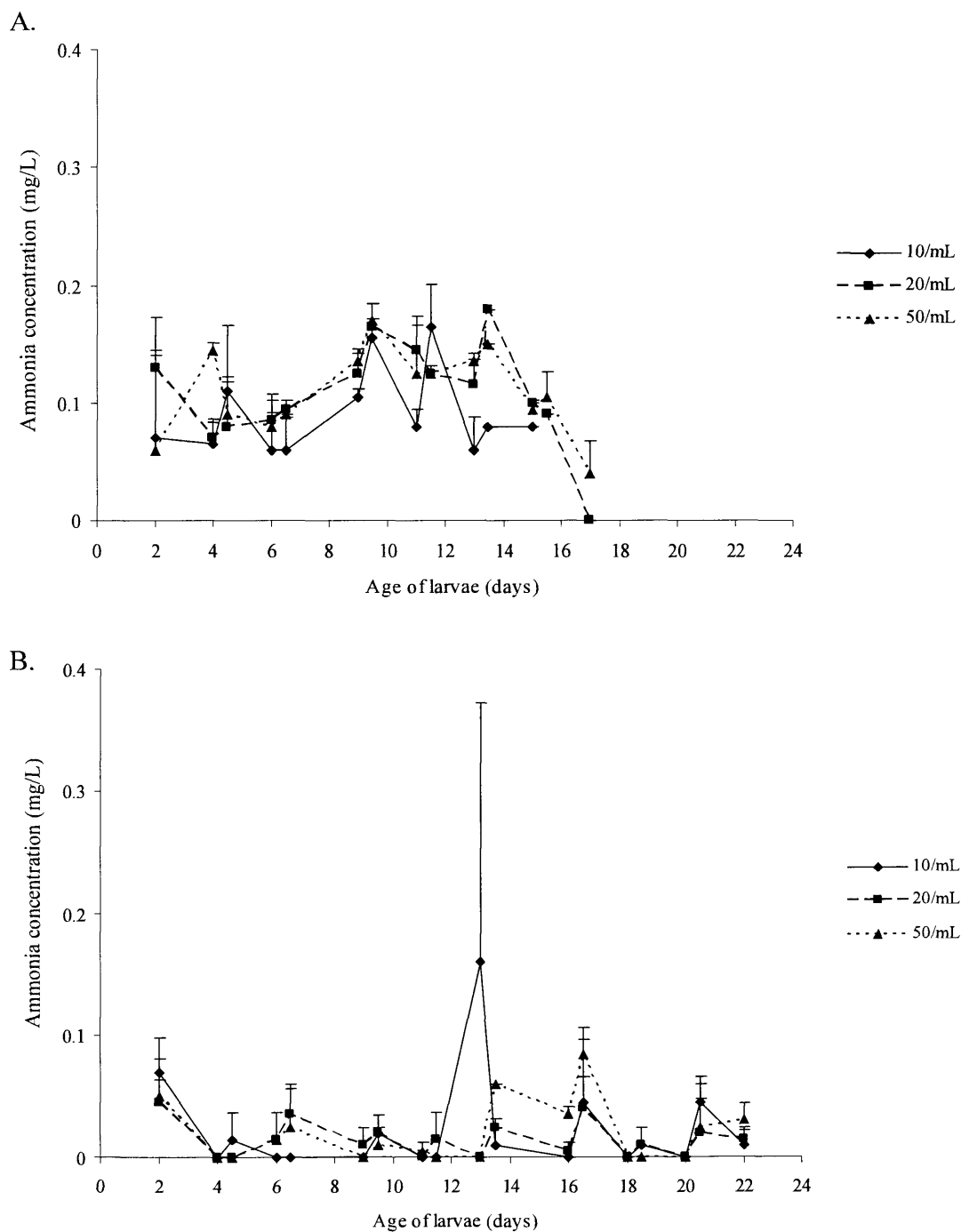


Figure 3.11. Concentrations of ammonia for stocking densities reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. $n=2$.

Chapter Four: Replication of results in flow-through culture: Assessments at two exchange rates

Introduction

Experiments on flow-through culture detailed in Chapter Three suggested variability between replicates. For example, one replicate at a stocking density of 20 larvae/mL reared at a flow rate equivalent to five exchanges of water/day had higher survival of larvae than the second replicate for that treatment (Fig. 3.2). A second example concerns cell consumption, which in Chapter Three differed between the controls in the first and second experiments (Fig. 3.3). Controls for the two experiments were stocked at the same density (10 larvae/mL) and reared with the same exchange rate (five exchanges of water/day), yet cell consumption in the control for the second experiment was as high as 300% greater than cell consumption in the first experiment (Fig. 3.3). It was expected that cell consumption would be similar between the controls, given the similarities in rearing conditions; the fact that they were not challenged how replicable flow-through culture in this system can be.

To address the above problem, factors such as survival, growth, cell consumption, duration of the competent period, and growth efficiency were examined with high replication (n=6) to assess the variability of, ostensibly, identical systems. These results were then used to gauge the results obtained in Chapter Three.

Methods

Set-up of the flow-through system

To assess the degree to which results obtained from the flow-through system of this thesis can be consistently replicated, the flow-through system previously set up at Oyster Seed Holdings (OSH) was re-activated during the summer of 2011. The same set-up and sources of brood stock outlined in Chapter Two were used. Oysters from the Aquaculture Genetics and Breeding Technology Center (ABC) hANA line were strip spawned to obtain gametes, and fertilized eggs were incubated for two days in a 4700 L tank. Six flow-through cultures were stocked with two-day-old larvae at 10 larvae/mL for two experiments testing exchange rate. The first experiment reared larvae at five exchanges of water/day and the second experiment reared larvae at ten exchanges of water/day.

For five exchanges of water/day, 56 females were strip spawned to obtain 50.6 million eggs, which were fertilized using three males. For ten exchanges of water/day, 100 females were strip spawned to obtain 300 million eggs. Eggs were fertilized with between 30 – 40 males.

Metrics for larvae

Procedures outlined in Chapter Three regarding tank draining, counting and measuring larvae, and harvest of competent larvae were followed in Chapter Four.

Feeding

Procedures for regulating input densities and cell selection of algae (*Pavlova lutherii*, *Isochrysis* sp., *Chaetoceros* sp., and *Tetraselmis suecica*) as outlined in Chapter Three were followed in Chapter Four.

Water Quality

Temperature, salinity, and pH were measured using the same instruments and procedures as outlined in Chapter Three. Based on results from the summer 2010 experiment (Chapter Three), it was determined that daily sampling for ammonia and bacteria was unnecessary, so sampling was reduced to once per week. Sampling for ammonia and bacteria began on the first day larvae were introduced to flow-through cultures and occurred on the same day every week thereafter. The same sampling procedures for ammonia and bacteria as outlined in Chapter Three were followed. If either ammonia or bacteria levels were higher than ammonia and bacteria levels obtained from the summer 2010 experiments, sampling was performed the next day to determine if the high reading was the beginning of a pattern or an isolated event.

Statistical Analysis

One way to understand the variation of a data set is to calculate the coefficient of variation (CV). The smaller the CV, the smaller the variation in the data. CV is normally expressed as a percent and allows comparisons to be made between different data sets. CV is calculated via the following equation:

$$CV (\%) = \frac{\sigma}{\mu}$$

where:

σ = standard deviation of the data set

μ = mean of the data set

Flow-through cultures were sampled three times per week, so separate CV calculations were performed for each day; therefore, CVs for the variables of each experiment are reported as a range.

To provide a statistical comparison between variation for five and ten exchanges of water/day, a Fisher's Test was performed. The Fisher's Test compares the variance of two sets of data, and is calculated using the following equation:

$$F_{\text{calc}} = \frac{s_1^2}{s_2^2}$$

where:

F_{calc} = calculated Fisher's value

s_1^2 = variance (standard deviation squared) of variable 1

s_2^2 = variance (standard deviation squared) of variable 2

Once F_{calc} is calculated, the value is compared with the critical F-value (F_{crit}) found in a standard F-test statistical table. If $F_{\text{calc}} < F_{\text{crit}}$, there is no significant difference between the variances of the two sets of data.

Results

Examining the replication of flow-through culture

Survival

At five and ten exchanges of water/day survival declined as larvae aged. Mean proportion of larvae (six replicates) that survived to the first day of harvest of competent larvae was 0.59 ± 0.19 (95% CI) on day 13 for five exchanges of water/day and 0.46 ± 0.13 (95% CI) on day 15 for ten exchanges of water/day (Fig. 4.1). There was no significant difference in survival between the two exchange rates ($p < 0.05$).

Over the course of larval development, the range for CV for survival was smaller at five exchanges of water/day, ranging from 20 – 28%, than at ten exchanges of water/day, ranging from 13 – 35% (Table 4.1). There was no significant difference in variance for survival between the two exchange rate experiments ($p > 0.05$).

Growth

Veligers reared at five exchanges of water/day grew from a mean of 76 – 217 μm over a period of 13 days (six replicates). Veligers reared at ten exchanges of water/day grew from a mean of 70 – 203 μm over a period of 15 days (six replicates). There was no significant difference in growth rate of larvae between the two exchange rates (Fig. 4.2; $p > 0.05$). Mean length of veligers (six replicates) on the first day competent larvae were harvested was 217 μm for five exchanges of water/day and 203 μm for ten exchanges of water/day. Mean length of veligers on the first day competent

larvae were harvested was significantly different between the two exchange rates (Fig. 4.2; $p < 0.05$). Larvae reared at five exchanges of water/day began to enter the competent stage at day 13, while competent larvae did not appear until day 15 for ten exchanges of water/day (Fig. 4.2).

The CV for length for each day of larval development was calculated from the means of the six flow-through tanks combined. For the six tanks reared at five exchanges of water/day, CV for length experienced a gradual increase over the course of larval development, from 1 – 8%, peaking on day 11 when the CV was 8% (Table 4.1). Among the six tanks reared at ten exchanges of water/day, CV for length also experienced a gradual increase over the course of larval development, from 0.3 – 9%, peaking on day 11 when the CV was 9% (Table 4.1). There was a significant difference in variance for length on days 2 and 4 between the two exchange rates ($p < 0.05$). For the remaining days, there was no significant difference in variance for length between the two exchange rates ($p > 0.05$).

The CV for length was calculated for each individual flow-through tank using the 35 larvae measured from each culture. The CVs for length within each tank reared at five exchanges of water/day increased as larvae aged, ranging from 5 – 23%, peaking around days 9 – 11 before declining. Individual CVs for each day and tank for larvae reared at five exchanges of water/day are reported in Table 4.2. The CVs for length within each tank reared at ten exchanges of water/day increased as larvae aged, from 0 – 30%. All six flow-through tanks reared at ten exchanges of water/day had a peak in CV occur around day 11 before declining. Individual CVs for each day and tank for larvae reared at ten exchanges of water/day are reported in Table 4.3.

Cell consumption and growth efficiency

Mean cell consumption (six replicates) increased over time for both exchange rates, with cell consumption increasing from 11,399 – 65,527 cells/larva/day for larvae reared at five exchanges of water/day, and from 12,419 – 69,619 cells/larva/day for larvae reared at ten exchanges of water/day (Fig. 4.2). Larvae reared at ten exchanges of water/day consumed significantly more cells overall than larvae reared at five exchanges of water/day (Fig. 4.2; $p < 0.05$).

The CV for cell consumption over time was smaller for five exchanges of water/day, which ranged from 22 – 52%, than for ten exchanges of water/day, which ranged from 35 – 75% (Table 4.1). There was no pattern to the fluctuation of CV over time in either experiment. Variance in cell consumption was significantly different between the two exchange rates on days 2 and 13 ($p < 0.05$). For the remaining days, there was no significant difference in variance for cell consumption between exchange rates ($p > 0.05$).

Mean growth efficiency (six replicates) for larvae reared at five exchanges of water/day ranged from 3 – 8.5% over the course of larval development. Mean growth efficiency (six replicates) for larvae reared at ten exchanges of water/day ranged from 1.7 – 7.6% over the course of larval development. Growth efficiency followed a similar pattern for both exchange rates – it was high on day 2, then experienced a drop on day 4, after which growth efficiency gradually increased as larvae aged. Mean growth efficiency was significantly higher overall during the course of larval development for larvae reared at five exchanges of water/day than at ten exchanges of water/day (Fig. 4.3; $p < 0.05$).

The CV for growth efficiency was smaller at five exchanges of water/day, which increased from 9 – 47% over the course of larval development, than at ten exchanges of water/day, which increased from 12 – 62% over the course of larval development (Table 4.1). There was a significant difference in variance for growth efficiency on day 9 between the two exchange rates ($p < 0.05$). For the remaining days, there was no significant difference in variance between the two exchange rates ($p > 0.05$).

Characteristics of the competent stage

At five exchanges of water/day, harvest of competent larvae occurred from day 13 to day 20. The mean length of competent larvae on the first day of harvest equaled 311 μm (six replicates) (Fig. 4.2). At ten exchanges of water/day, harvest of competent larvae occurred from day 15 to day 21, with a mean length of competent larvae equaling 291 μm (six replicates) on the first day of harvest (Fig. 4.2). Mean duration of the competent period (six replicates) for five exchanges of water/day was five days, and mean duration of the competent period (six replicates) for ten exchanges of water/day was seven days. There was no significant difference in rate of harvest or duration of the competent period between the two exchange rates (Fig. 4.4; Fig. 4.5; $p > 0.05$).

The harvest of competent larvae had three distinct trends at five exchanges of water/day (Fig. 4.5). Two cultures had competent larvae harvested several days before the other four cultures. Two cultures experienced peak harvest of competent larvae on the first day of harvest, followed by a linear decline in harvest. The last two cultures had the latest peak in harvest of competent larvae (Fig. 4.5). At ten exchanges of water/day, there was greater similarity among cultures when competent larvae were harvested –

beginning and end of the harvest occurred on the same days for all tanks. There was also a greater coincidence of when peak harvest of competent larvae occurred (Fig. 4.5).

The range for CV was larger for cumulative harvest of competent larvae for five exchanges of water/day, ranging from 45 – 155% as larvae aged, than for ten exchanges of water/day, which ranged from 7 – 116% as larvae aged (Table 4.1). There was no pattern for the CV for five exchanges of water/day, but for ten exchanges of water/day CV was highest on the first day competent larvae were harvested, day 15, and then declined with each subsequent harvest. Variance for cumulative harvest of competent larvae differed significantly between the two exchange rates on the second day of harvest (day 15 for five exchanges of water/day, day 17 for ten exchanges of water/day) ($p < 0.05$). There was no significant difference in variance for cumulative harvest for the remaining harvest days between five and ten exchanges of water/day ($p > 0.05$).

Flow-through cultures reared at five exchanges of water/day had a mean harvest of competent larvae (six replicates) of two million. Flow-through cultures reared at ten exchanges of water/day had a mean harvest of competent larvae (six replicates) of one million (Table 4.4). Cultures reared at five exchanges of water/day had significantly greater survival of larvae from day 2 to the last day of harvest, 57%, than flow-through cultures reared at ten exchanges of water/day, 24% (Table 4.4; $p < 0.05$).

Ranking parameters by CV

The CV represents the amount of variation within a data set. A large CV indicates there is large variation among data points, as does a large range among the values themselves. Ranking the variables from lowest to highest CV, the order is the same for both five and ten exchanges of water/day (Table 4.1):

1. Length
2. Survival
3. Cell consumption
4. Growth efficiency
5. Cumulative harvest of competent larvae

Water Quality

Temperature at five exchanges of water/day ranged from 24.2 – 25.9°C (Fig. 4.6). Salinity ranged from 8.6 – 10 (Fig. 4.7). The pH meter was broken during the experiment at five exchanges of water/day, so pH was not recorded. At ten exchanges of water/day, temperature ranged from 25.3 – 28.4°C (Fig. 4.6). Salinity ranged from 8.3 – 12.5 (Fig. 4.7) and pH ranged from 7.6 – 7.7 (Fig. 4.8).

Concentrations of ammonia at five and ten exchanges of water/day remained below 0.1 mg/L except on day 16 when concentrations of ammonia exceeded 0.15 mg/L in one culture. The concentration was deemed high compared to previous ammonia levels, so ammonia was measured again from that culture on the following day (day 17). By day 17, ammonia had dropped to 0.08 mg/L (Fig. 4.9). Growth of bacteria was only observed at five exchanges of water/day. Growth of bacteria occurred on day 2, and only in half the flow-through cultures (Table 4.5).

Comparing results from Chapter Four with Chapter Three

Mean survival proportion of larvae reared at five exchanges of water/day to the first day of harvest of competent larvae ranged from 0.40 – 0.78, as established in Chapter Four (Fig. 4.1). In Chapter Three, mean survival proportion of larvae in the control for the first experiment, reared at five exchanges of water/day, was 0.36. For the

control in the second experiment, also reared at five exchanges of water/day, mean survival proportion to the first day of harvest of competent larvae was 0.55 (Fig. 3.1). The CV for survival, as established in Chapter Four, ranged from 20 – 28%. The CV for survival in the first experiment reared at five exchanges of water/day in Chapter Three ranged from 0.3 – 44% over the course of larval development. In the second experiment in Chapter Three, also reared at five exchanges of water/day, CV for survival ranged from 11 – 75% over the course of larval development (Table 4.6).

Mean length of veligers on the first day of harvest of competent larvae reared at five exchanges of water/day was 217 μm , as established in Chapter Four (Fig. 4.2). In Chapter Three, mean length of veligers for the control in the first experiment, reared at five exchanges of water/day, was 242 μm . In the second experiment in Chapter Three, also reared at five exchanges of water/day, mean length of veligers for the control was 210 μm (Fig. 3.3). The range for CV for length, as established in Chapter Four, was 1 – 8% over the course of larval development. The CV for length in the first experiment in Chapter Three, reared at five exchanges of water/day, ranged from 1 – 10% over the course of larval development. In the second experiment in Chapter Three, also reared at five exchanges of water/day, CV for length ranged from 0 – 10% over the course of larval development (Table 4.6).

Mean cell consumption of larvae reared at five exchanges of water/day ranged from 11,399 – 65,527 cells/larva/day over the course of larval development, as established in Chapter Four. In Chapter Three, mean cell consumption of larvae for the control in the first experiment, reared at five exchanges of water/day, ranged from 8,800 – 38,094 cells/larva/day over the course of larval development. Mean cell consumption

of larvae in the control in the second experiment, also reared at five exchanges of water/day, ranged from 21,168 – 143,344 cells/larva/day over the course of larval development (Fig. 3.3). The CV for cell consumption, as established in Chapter Four, ranged from 22 – 52% over the course of larval development. In the first experiment in Chapter Three, reared at five exchanges of water/day, the CV for cell consumption ranged from 1 – 83% over the course of larval development. In the second experiment, also reared at five exchanges of water/day, the CV for cell consumption ranged from 0.9 – 61% over the course of larval development (Table 4.6).

Mean growth efficiency of larvae reared at five exchanges of water/day ranged from 3 – 8.5%, as established in Chapter Four. In Chapter Three, growth efficiency for the control in the first experiment, reared at five exchanges of water/day, ranged from 4.9 – 8.7% over the course of larval development. For the control in the second experiment, also reared at five exchanges of water/day, growth efficiency ranged from 0.7 – 4% over the course of larval development (Fig. 3.4). The CV for growth efficiency, as established in Chapter Four, ranged from 9 – 47% over the course of larval development. In Chapter Three, the CV for growth efficiency in the first experiment, reared at five exchanges of water/day, ranged from 0.5 – 56% over the course of larval development. For the second experiment in Chapter Three, also reared at five exchanges of water/day, CV for growth efficiency ranged from 24 – 141% over the course of larval development (Table 4.6).

Mean harvest of competent larvae reared at five exchanges of water/day was two million, as established in Chapter Four. Mean survival from day two to the last day of harvest was 57%, as established in Chapter Four. In Chapter Three, mean harvest of competent larvae for the control in the first experiment, reared at five exchanges of

water/day, was 1.4 million and mean survival of larvae from day 2 to the last day of harvest was 32%. Mean harvest of competent larvae for the control in the second experiment, also reared at five exchanges of water/day, was 1.8 million and mean survival of larvae from day 2 to the last day of harvest was 41% (Table 3.1; Table 3.2). The CV for cumulative harvest of competent larvae, as established in Chapter Four, ranged from 45 – 155% over the course of larval development. In Chapter Three, the CV for cumulative harvest of competent larvae in the first experiment, reared at five exchanges of water/day, ranged from 8 – 88% over the course of larval development. In the second experiment, also reared at five exchanges of water/day, CV for cumulative harvest of competent larvae ranged from 1 – 46% over the course of larval development (Table 4.6).

The ranges for CVs were calculated for the controls in Chapter Three (Table 4.6). The order, from lowest to highest, for CV for the first experiment in Chapter Three resulted in the following ranking:

1. Length
2. Survival
3. Growth efficiency
4. Cumulative harvest of competent larvae
5. Cell consumption

The order, from lowest to highest, for CV for the second experiment in Chapter Three resulted in the following ranking:

1. Length
2. Cumulative harvest of competent larvae

3. Cell consumption
4. Survival
5. Growth efficiency.

The ranking order for both exchange rates for Chapter Three differs from the ranking order established in Chapter Four: length, survival, cell consumption, growth efficiency and cumulative harvest of competent larvae (Table 4.1). The ranges for CV obtained from the control in Chapter Three are generally larger than the ranges for CV in Chapter Four, with the exception of length.

Discussion

Examining the replication of flow-through culture

Survival

Survival from day two to the first day of harvest of competent larvae for the flow-through system was established in Chapter Four at 0.59 ± 0.19 (95% CI) at 10 larvae/mL and five exchanges of water/day. At 10 larvae/mL and ten exchanges of water/day, survival for this flow-through system was established in Chapter Four at 0.46 ± 0.13 (95% CI) (Fig. 4.1). Five or ten exchanges of water/day can be used without significantly affecting survival to the first day of harvest for this flow-through system.

Survival proportion had the second lowest variation as indicated by CV for both exchange rates, indicating variation will be similar among tanks regardless of exchange rate. There is also less variance in survival when larvae are reared at the lower exchange rate, as five exchanges of water/day had the smaller range for CV (Table 4.1). Future experiments using the same flow-through system may be unable to monitor all aspects of flow-through culture, in which case monitoring survival would provide a means of indirectly monitoring the health of larvae. A flow-through culture experiencing lower survival than the other cultures in the flow-through system may be an indicator of a problem within that culture and warrant further investigation.

Growth

There was no significant difference in growth rate between the two exchange rates, yet larvae reared at five exchanges of water/day reached the first day of harvest of

competent larvae in a shorter amount of time (13 days) than larvae reared at ten exchanges of water/day (15 days) (Fig. 4.2). With no significant difference in growth rate, one might expect that harvest of competent larvae would begin on the same day for both exchange rates. The fact that it did not may be the result of incorrect protocol used to harvest competent larvae. The criterion used for determining harvest of competent larvae was the appearance of a pigmented eye spot, leading to the harvest of competent larvae with mean lengths of 311 μm (five exchanges of water/day) and 291 μm (ten exchanges of water/day) in length. Competent larvae may have been harvested too early, as evidenced by their small size, since mean size of competent larvae ranges from 290 – 360 μm (Fitt et al., 1990; Talmage and Gobler, 2009). Larvae harvested from Chapter Four are near the smaller end of the given size range for competent larvae. Mean length of veligers was also small: 217 μm at five exchanges of water/day and 203 μm at ten exchanges of water/day, further implying that the population was not ready to be harvested. Waiting for the appearance of a crawling foot may have resulted in competent larvae being harvested at the same time in the two experiments rather than at different times, and might have resulted in larger lengths for veligers and competent larvae.

For length, the range for CV was lowest out of all the other variables at both five and ten exchanges of water/day, indicating that length may be the least sensitive to different exchange rates. There was less variation in length when larvae were reared at five exchanges of water/day rather than at ten exchanges of water/day as evidenced by the smaller range for CV for length at the lower exchange rate (Table 4.1). The size of larvae is another direct indicator of their health. It follows that the greater the

consistency among cultures when measuring length, the easier it will be to notice problems within an individual culture if growth starts to go awry.

Size variation was compared within each individual culture versus among the six flow-through cultures combined. Variation in length of larvae was greater within an individual culture, indicating that larvae grew and developed at different rates within each tank. Variation also increased with larval age (Table 4.2; Table 4.3). The increase in variation for length with age within flow-through culture is most likely a product of the individual development rates of larvae. The harvest of competent larvae took several days for all flow-through cultures, indicating that some larvae grew and developed faster than others (Fig. 4.5). The divergence in size became greater as larvae aged, perhaps as a result of competition between the larger and smaller larvae for food. While food was not a limiting factor in the flow-through system, the increased cell consumption of larger larvae may have limited access to the algae of the smaller larvae. As the larger larvae continued to consume more cells at a faster rate, access to the algae would have been limited further for the smaller larvae, increasing the divergence in size.

Cell consumption and growth efficiency

Cell consumption was significantly greater overall at ten exchanges of water/day, which ranged from 12,419 – 69,619 cells/larva/day than at five exchanges of water/day, which ranged from 11,399 – 65,527 cells/larva/day; however, the range for both exchange rates was comparable (Fig. 4.2). Cell consumption at five and ten exchanges of water/day in Chapter Four had the third largest range for CV, indicating greater variation in cell consumption than other parameters. Variance for cell consumption at ten exchanges of water/day is significantly greater on day 2 and day 13 than variance for cell

consumption at five exchanges of water/day, but cell consumption does not differ significantly between the two exchange rates on either of these days. The significant difference in variance may be an example of variation that can be expected to occur within flow-through cultures in the flow-through system of this thesis.

At five exchanges of water/day larvae converted the food they consumed to biomass with greater efficiency (3 – 8.5%) than larvae at ten exchanges of water/day (1.7 – 7.6%), despite the greater cell consumption of larvae reared at ten exchanges of water/day. The greater growth efficiency probably accounted for the faster growth rate observed at the lower exchange rate.

Growth efficiency (the quotient of the change in biomass over total food consumption multiplied by 100) had the second to largest range for CV, which may be due to the nature of the growth efficiency calculation. Growth efficiency is subject to variance from total food consumption and biomass, which is calculated from the number of larvae in each culture. There was moderate variation in survival proportion and cell consumption in cultures from Chapter Four (Table 4.1); therefore, variability in survival proportion and cell consumption was probably magnified in the growth efficiency equation, enlarging the CV for growth efficiency.

Characteristics of the competent stage

Larvae reared at five exchanges of water/day had a shorter duration for the competent period (five days) than larvae reared at ten exchanges of water/day (seven days) (Fig. 4.4; Fig. 4.5). Once harvest of competent larvae began, rate of harvest between the two exchange rates was not significantly different, indicating that exchange rate did not result in a faster or slower harvest of competent larvae (Fig. 4.4). Survival of

larvae from day 2 to last day of harvest of competent larvae was greater at five exchanges of water/day, accounting for the greater harvest of competent larvae (Table 4.4).

The range for CV for cumulative harvest of competent larvae was the largest of the five variables measured and was the only variable where the range for CV was larger at five exchanges of water/day than at ten exchanges of water/day (Fig. 4.5; Table 4.1). Given that there may be instances when harvest of competent larvae varies widely among cultures (as seen at five exchanges of water/day) or instances when harvest of competent larvae is similar among cultures (as seen at ten exchanges of water/day), it seems that harvest of competent larvae from this flow-through system is less predictable than other variables.

The range for CV for all variables except cumulative harvest of competent larvae was smaller at five exchanges of water/day, suggesting that a lower exchange rate reduces variation among cultures in this flow-through system (Table 4.1). For cumulative harvest of competent larvae at five exchanges of water/day, the large variation was due to the differences in harvest of competent larvae among flow-through cultures (Fig. 4.5).

Comparing results established in Chapter Four with results from Chapter Three

Survival for the control in the first experiment in Chapter Three, reared at five exchanges of water/day, was outside the range of means established in Chapter Four. Survival proportion was 0.36, and the established range was 0.4 – 0.78. In the second experiment in Chapter Three, also reared at five exchanges of water/day, survival proportion was 0.55, within the range established in Chapter Four. It is difficult to know what may have caused the low survival in the first experiment, as development of the

larvae did not seem to be impaired. Cell consumption, growth, and growth efficiency for the first experiment were similar to the established cell consumption, growth, and growth efficiency in Chapter Four. No bacteria growth was observed in the first experiment in Chapter Three, and temperature and salinity were similar between Chapters Three and Four (Fig. 3.8; Fig. 3.9; Fig. 4.6; Fig. 4.7), so it is unlikely that these factors negatively influenced survival. There are likely other sources of variation that contributed to low survival of the control in Chapter Three that were not examined, as it was impossible to monitor every minute aspect of the flow-through system for this thesis.

Mean length of veligers on the first day competent larvae were harvested from the control in the first experiment in Chapter Three, reared at five exchanges of water/day, were larger (242 μm) than the length established in Chapter Four (217 μm). Mean length of veligers on the first day competent larvae were harvested from the control in the second experiment in Chapter Three, also reared at five exchanges of water/day, was similar (210 μm) to the established length (217 μm). Protocol error when harvesting competent larvae may account for the size discrepancy between the established veliger length in Chapter Four and the veliger length in the first experiment. The harvest of competent larvae may have been premature in Chapter Four, as the mean length of competent larvae was 311 μm , on the smaller side for competent larvae, which usually range between 290 – 360 μm (Fitt et al., 1990; Talmage and Gobler, 2009). Larvae reared in the first and second experiments in Chapter Three were also on the small end of the competent size range, measuring 306 μm and 304 μm , respectively, suggesting that harvest of competent larvae may have been premature in Chapter Three as well.

Cell consumption for the control in the first experiment in Chapter Three, reared at five exchanges of water/day, ranged from 8,800 – 38,094 cells/larva/day and fell within the range for cell consumption established in Chapter Four (11,399 – 65,527 cells/larva/day) (Fig. 3.3; Fig. 4.2). Cell consumption for the control in the second experiment in Chapter Three, also reared at five exchanges of water/day, ranged from 21,168 – 143,344 cells/larva/day and exceeded the cell consumption range established in Chapter Four; therefore the latter estimates for cell consumption may have been an anomaly. The anomalous data for cell consumption may be due to superfluous feeding by larvae, which occurs when cell consumption is high but assimilation of the food consumed by the organism is low (Fowler and Fisher, 1983). Superfluous feeding occurs when food is in excess, so continuously feeding organisms consume large quantities of food, but the particles pass through the gut so rapidly that they are not effectively digested (Doohan, 1973). An error when allocating food to the flow-through tanks could have resulted in excess food being present in the tank, thus inducing superfluous feeding. The feeding error could have been propagated through multiple days, resulting in the large cell consumption observed in the second experiment in Chapter Three. The possibility also exists that an error in counting the algae cells was made during the experiment.

Growth efficiency established in Chapter Four for larvae reared at five exchanges of water/day was between 3 – 8.5%. Growth efficiency for the control in the first experiment in Chapter Three, reared at five exchanges of water/day, was similar to the growth efficiency established in Chapter Four, ranging from 4.9 – 8.7%. Growth efficiency for the control in the second experiment in Chapter Three, also reared at five

exchanges of water/day, ranged from 0.7 – 4%. The range for growth efficiency in the second experiment in Chapter Three was smaller than the range for growth efficiency established in Chapter Four. Since cell consumption factors into the growth efficiency calculation, and cell consumption for the control in the second experiment in Chapter Three was high, this would have resulted in a smaller quotient for growth efficiency.

Mean duration of the competent period for the control in the first experiment in Chapter Three, reared at five exchanges of water/day, was four days, similar to the five day duration of the competent period established in Chapter Four. For the control in the second experiment in Chapter Three, also reared at five exchanges of water/day, mean duration of the competent period was seven days, a couple days longer than the duration of the competent period established in Chapter Four. The longer duration of the competent period for the control in the second experiment may be due to slower growth. Competent larvae were not observed until day 16 in the second experiment in Chapter Three, while competent larvae were observed on day 11 in Chapter Four. Larvae were growing and developing slower in the second experiment in Chapter Three, which may also account for the longer duration of the competent period.

Mean harvest of competent larvae established in Chapter Four was two million, and competent larvae harvested from both experiments in Chapter Three were similar – 1.4 million from the control in the first experiment in Chapter Three, and 1.8 million from the second experiment in Chapter Three. Despite the slower growth and longer duration of the competent period experienced during the second experiment in Chapter Three, total harvest of competent larvae was similar to that found among the highly replicated work in Chapter Four.

The survival of larvae from day 2 to the last day of harvest of competent larvae established in Chapter Four was 57%. Survival obtained from the controls in the first and second experiments in Chapter Three, both reared at five exchanges of water/day, were 32% and 41%, respectively, lower than the survival established in Chapter Four. Survival to the first day competent larvae were harvested was already low for the first and second experiments in Chapter Three, at 0.36 and 0.55, respectively, so it can be expected that mortality would continue during the harvesting period.

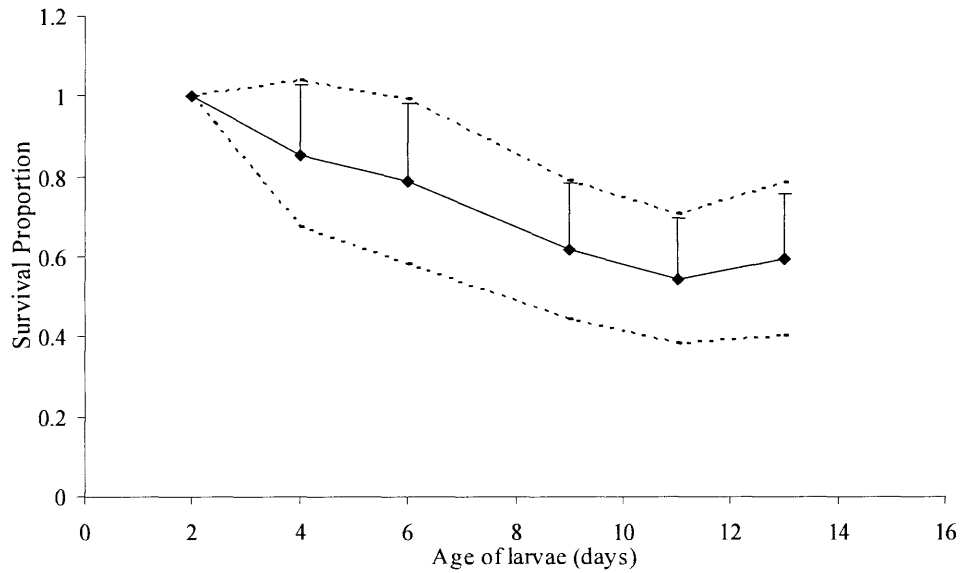
The ranges for CV for the controls in Chapter Three were larger than the ranges for CV established in Chapter Four, with the exception of length. Ranges for CV for length at five and ten exchanges of water/day coincided almost exactly with the ranges for CV for length in Chapter Four (Table 4.1; Table 4.6). Length had low variation, indicating that it will be fairly consistent if metabolic needs of larvae are met. The larger ranges for CV for the other variables measured in the first and second experiments in Chapter Three are probably a factor of having only two replicates assigned to each control, which would result in larger variation.

Conclusions

The larger number of replicates accomplished in this Chapter allows one to set expected values for flow-through larval culture, at least for the following variables: survival, length, cell consumption, growth efficiency, and characteristics of the competent stage. Larvae were reared at both five and ten exchanges of water/day, so expected values can be applied to both exchange rates. Variation was smaller among flow-through cultures reared at five exchanges of water/day than at ten exchanges of water/day, indicating that the lower exchange rate produced greater consistency among flow-through cultures. The lower the variation among cultures, the easier it will be to identify inconsistencies in survival, growth, etc of larvae. Survival and length, both direct indicators of health of larvae, had low variance at both five and ten exchanges of water/day, so any tank inconsistencies regarding these two measurements should indicate a problem with the culture.

Data for survival, length, cell consumption, growth efficiency, and the competent stage obtained from the controls in Chapter Three, were compared with the corresponding data established in Chapter Four. Comparisons with Chapter Four enabled the identification of anomalous data in Chapter Three. The only anomalous conditions identified in Chapter Three were associated with cell consumption (cells/larva/day).

A.



B.

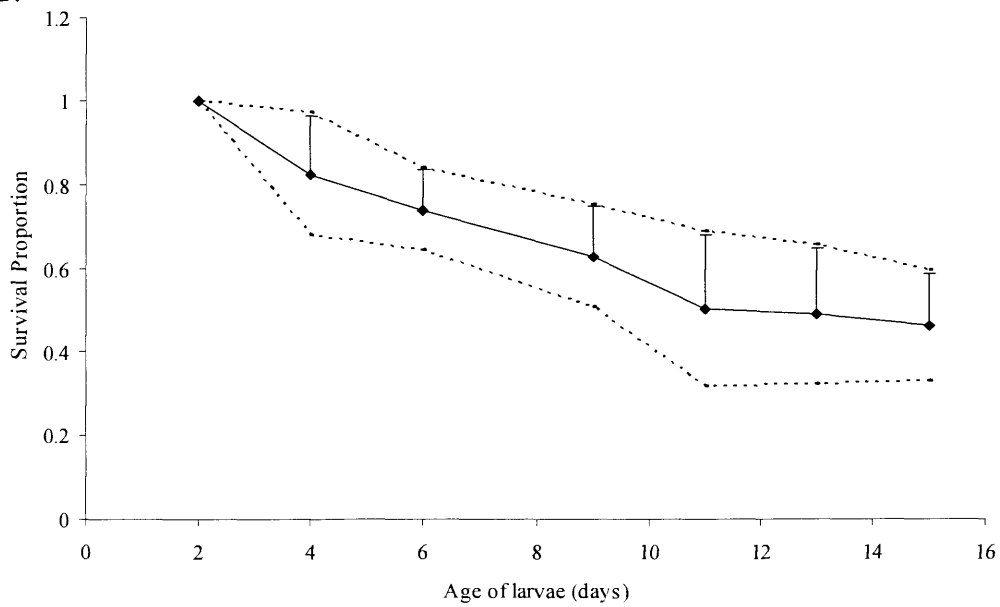


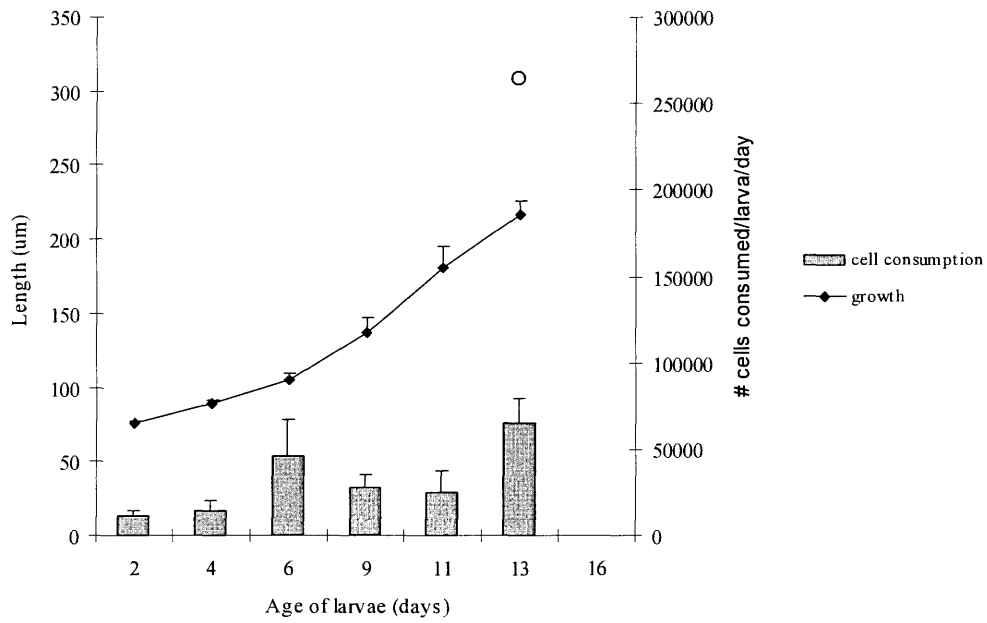
Figure 4.1 Mean survival proportion of tanks stocked at 10 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B). 95% confidence interval is represented by the dotted lines. Error bar = +1 sd. n=6.

Table 4.1. Coefficient of variations (%) for five variables measured for larvae reared at five and ten exchanges of water/day during the course of development. Flow-through cultures were stocked at 10 larvae/mL. Numbers in parenthesis indicate the order of ranges for coefficient of variation, from smallest to largest. Age of larvae is designated in the top row. The exchange rate (exchanges of water/day) is designated in the second row n=6.

Parameter	2		4		6		9		11		13	
	Five	Ten	Five	Ten	Five	Ten	Five	Ten	Five	Ten	Five	Ten
Length (1)	1	0.3	2	0.7	4	2	7	5	8	9	4	3
Survival (2)			20	17	25	13	27	19	28	35	27	33
Cell consumption (3)	24	75	42	41	46	44	28	35	52	43	22	69
Growth Efficiency (4)	9	12	20	32	18	30	45	23	47	47	22	62
Cumulative competent harvest (5)											45	

Parameter	15		16		17		18		19	
	Five	Ten	Five	Ten	Five	Ten	Five	Ten	Five	Ten
Length (1)		5								
Survival (2)		27								
Cell consumption (3)		50								
Growth Efficiency (4)		25	12							
Cumulative competent harvest (5)	155	116	27		51	23	14			7

A.



B.

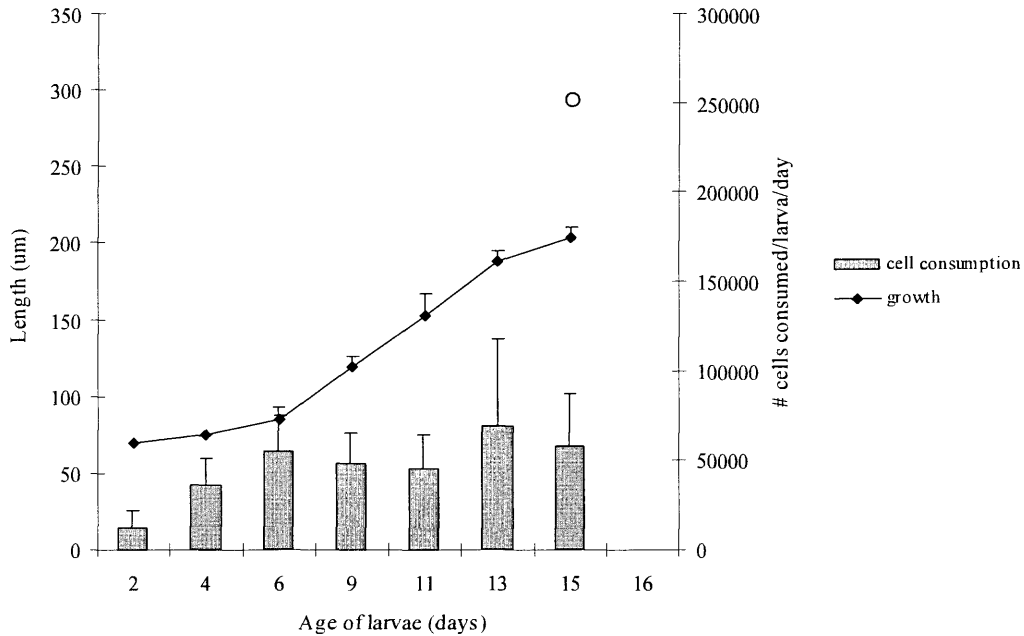


Figure 4.2. Mean length and cell consumption of tanks stocked at 10 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Harvesting of competent larvae began on day 13 at five exchanges of water/day and day 15 at ten exchanges of water/day. Mean size of competent larvae on first day of harvest is indicated by the open circles. Error bars = +1 sd. n=6.

Table 4.2. Mean lengths of larvae (μm) and coefficient of variation (% in parentheses) obtained during the course of development (days, top row) in six flow-through tanks stocked at 10 larvae/mL and reared at five exchanges of water/day. n=35

Tank	2	4	6	9	11	13
1	76.6 (5.4)	90.8 (6.6)	108.8 (13)	151.7 (18)	177.1 (19)	223.6 (16)
2	75.7 (5.4)	88.3 (6.6)	98.6 (11)	123.6 (21)	153.86 (20)	199.3 (20)
3	75.0 (6.7)	90.6 (7.2)	103.8 (10.)	138.6 (21)	188.8 (15)	219.6 (19)
4	76.7 (4.9)	92.0 (5.5)	111.3 (9.8)	129.0 (11)	178.8 (14)	217.1 (15)
5	75.7 (5.4)	86.7 (5.9)	103.6 (8.6)	143.1 (18)	194.6 (16)	215.1 (18)
6	77.1 (4.8)	87.1 (6.1)	102.7 (9.3)	136.7 (16)	192.0 (23)	220.8 (19)

Table 4.3. Mean lengths of larvae (μm) and coefficient of variation (% in parentheses) obtained during the course of development (days, top row) in six flow-through tanks stocked at 10 larvae/mL and reared at ten exchanges of water/day. n=35

	2	4	6	9	11	13	15
Tank							
1	70.0 (0.0)	75.3 (6.8)	85.0 (9.1)	126.4 (13)	152.0 (24)	184.0 (23)	206.3 (23)
2	70.1 (3.7)	74.4 (6.3)	84.7 (7.0)	113.6 (14)	137.1 (30)	180.3 (29)	198.8 (23)
3	70.0 (0.0)	75.1 (5.9)	82.7 (8.2)	114.3 (15)	144.7 (25)	194.4 (23)	193.1 (20)
4	69.7 (4.2)	76.0 (5.7)	88.4 (9.9)	128.6 (16)	174.0 (24)	189.4 (23)	207.4 (19)
5	70.3 (2.4)	75.0 (6.5)	86.0 (9.5)	118.0 (14)	145.7 (21)	196.6 (21)	200.3 (20.)
6	70.3 (2.4)	75.1 (6.7)	84.1 (11)	117.8 (16)	166.0 (23)	185.7 (27)	214.1 (16)

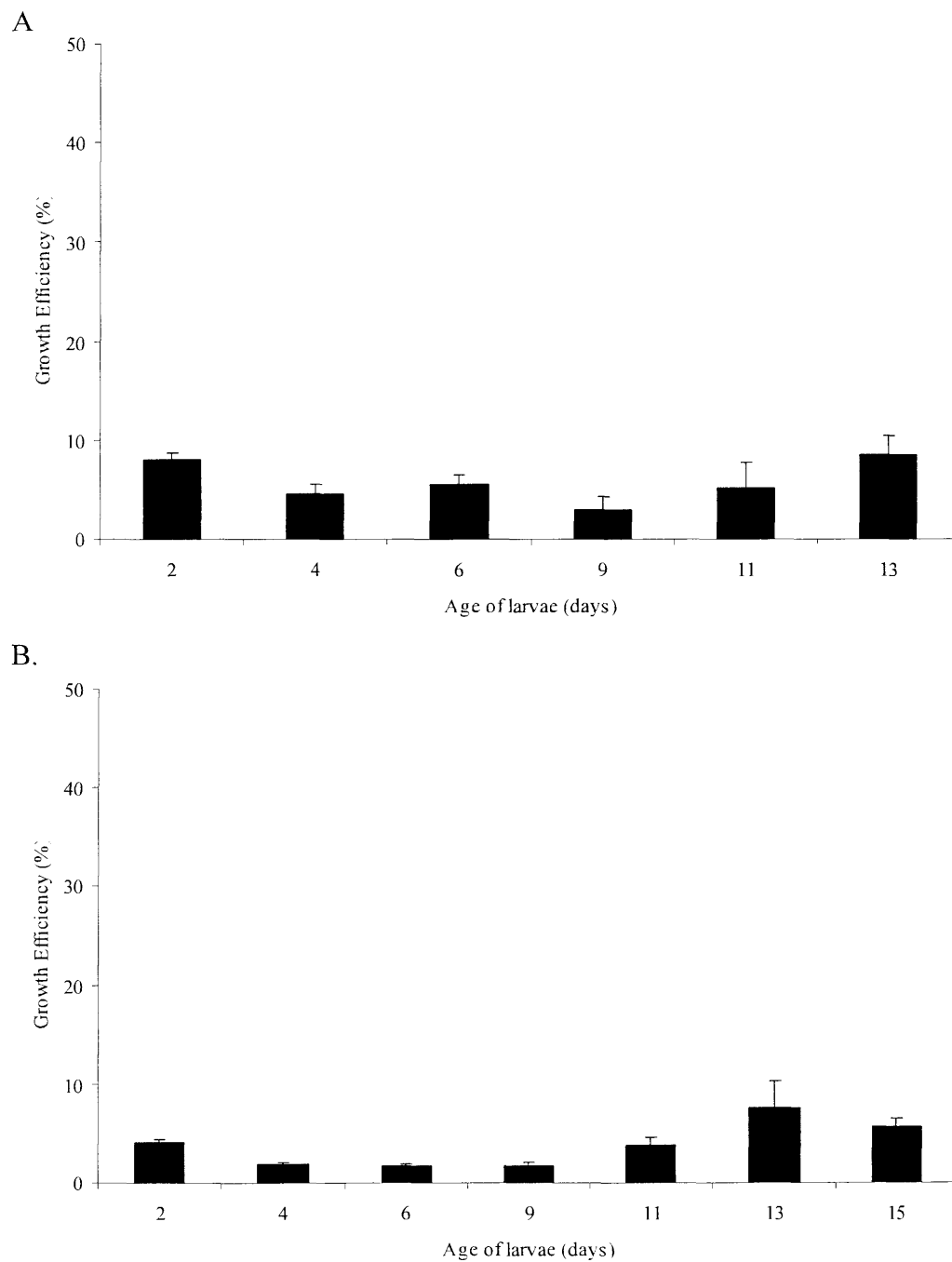


Figure 4.3. Mean growth efficiency for tanks stocked at 10 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars = +1 sd. n=6.

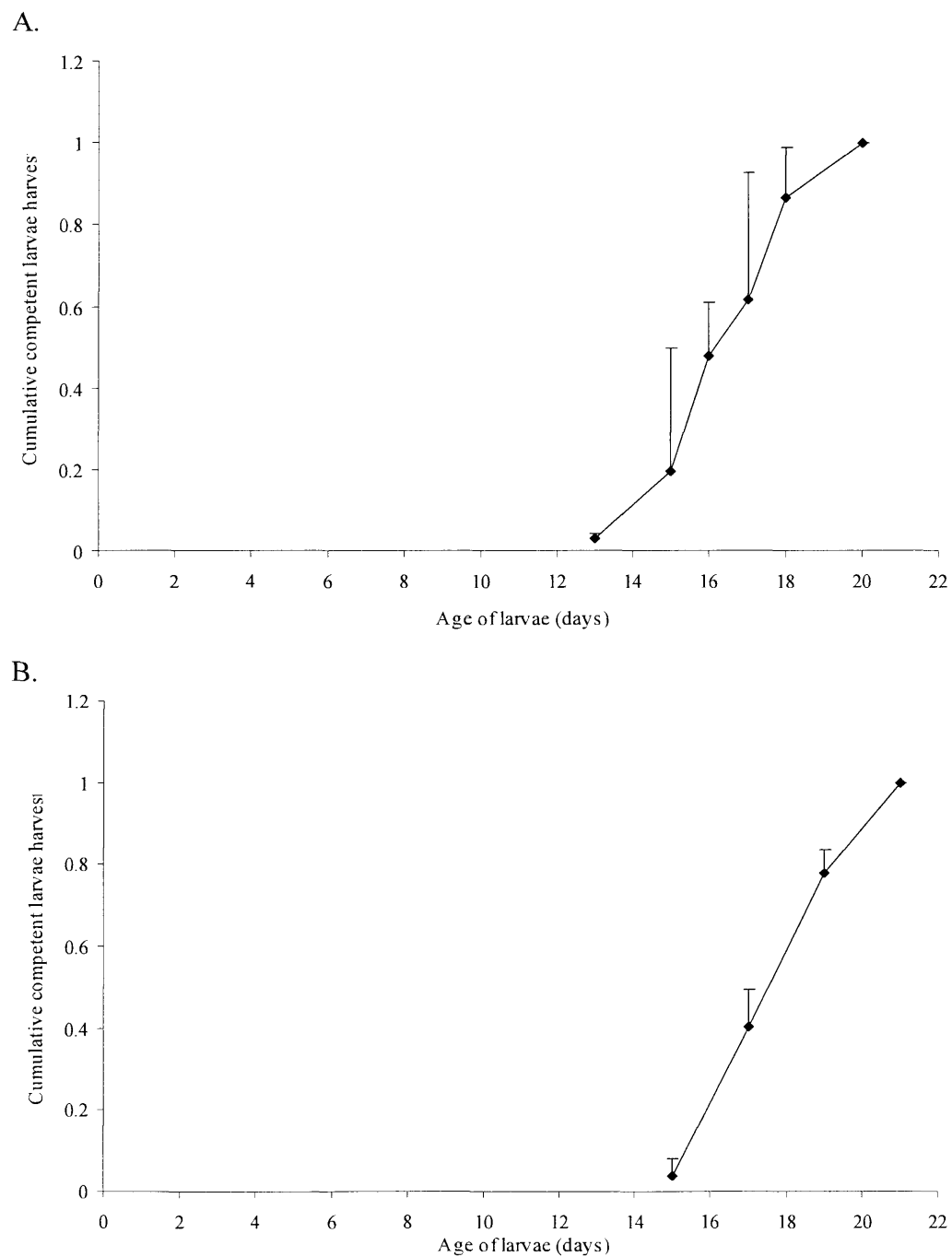
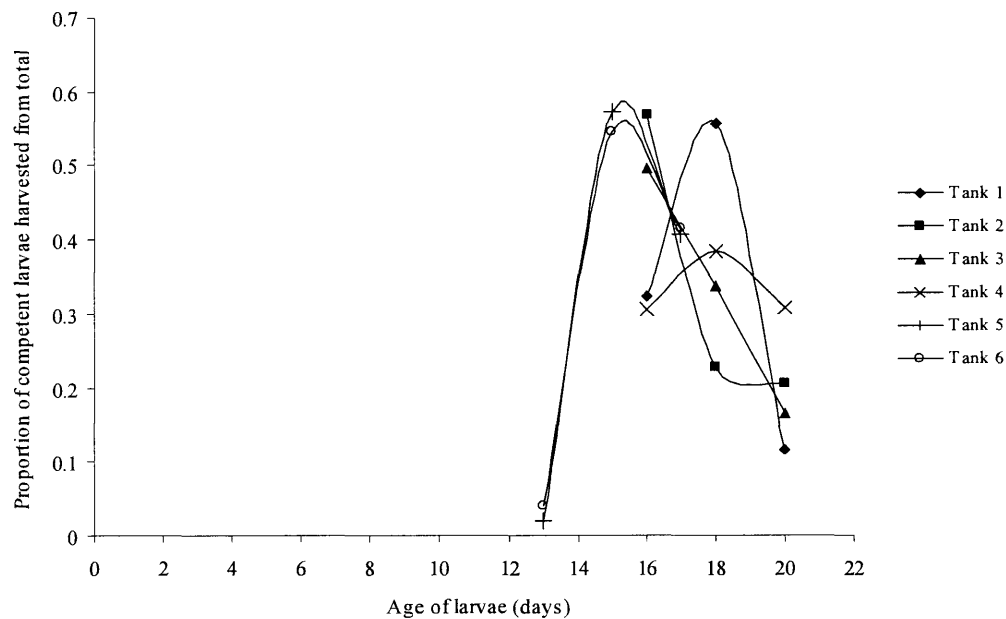


Figure 4.4. Mean cumulative harvest of competent larvae for tanks stocked at 10 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars = +1 sd. n=6.

A.



B.

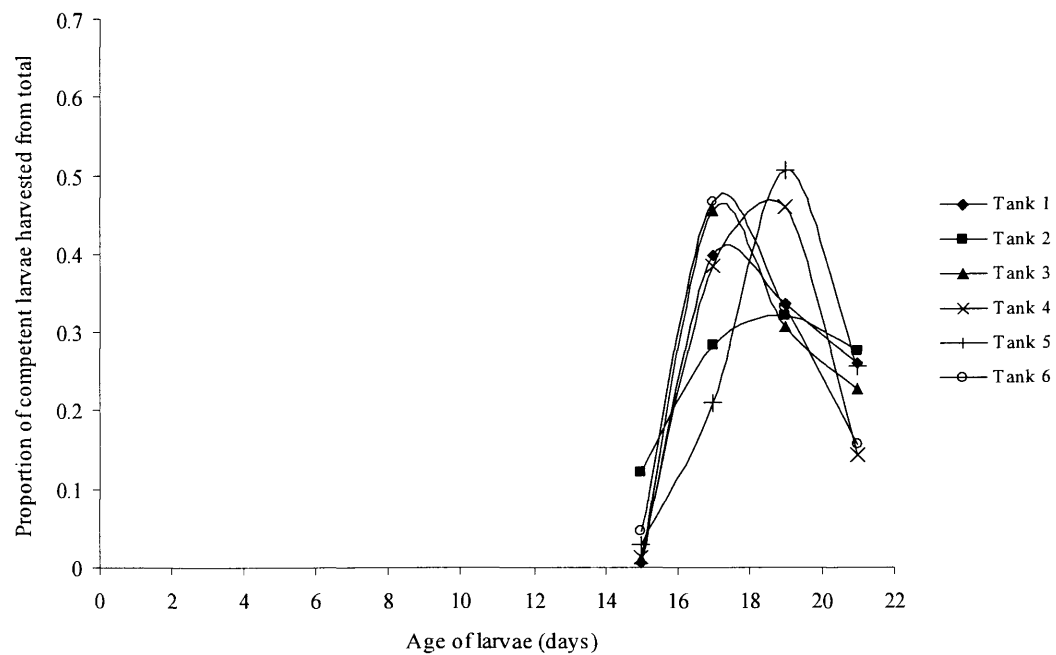


Figure 4.5. Harvest of competent larvae from flow-through cultures stocked at 10 larvae/mL and reared at five exchanges of water/day (A) and ten exchanges of water/day (B).

Table 4.4. Mean harvest of competent larvae from tanks stocked at 10 larvae/mL and reared at five and ten exchanges of water/day. Mean survival from day 2 to last day of harvest of competent larvae is provided. n=6

Tank exchanges/day	Total competent larvae harvested (millions)	Survival (%) from day 2 to last harvest day
Five	2	57
Ten	1	24

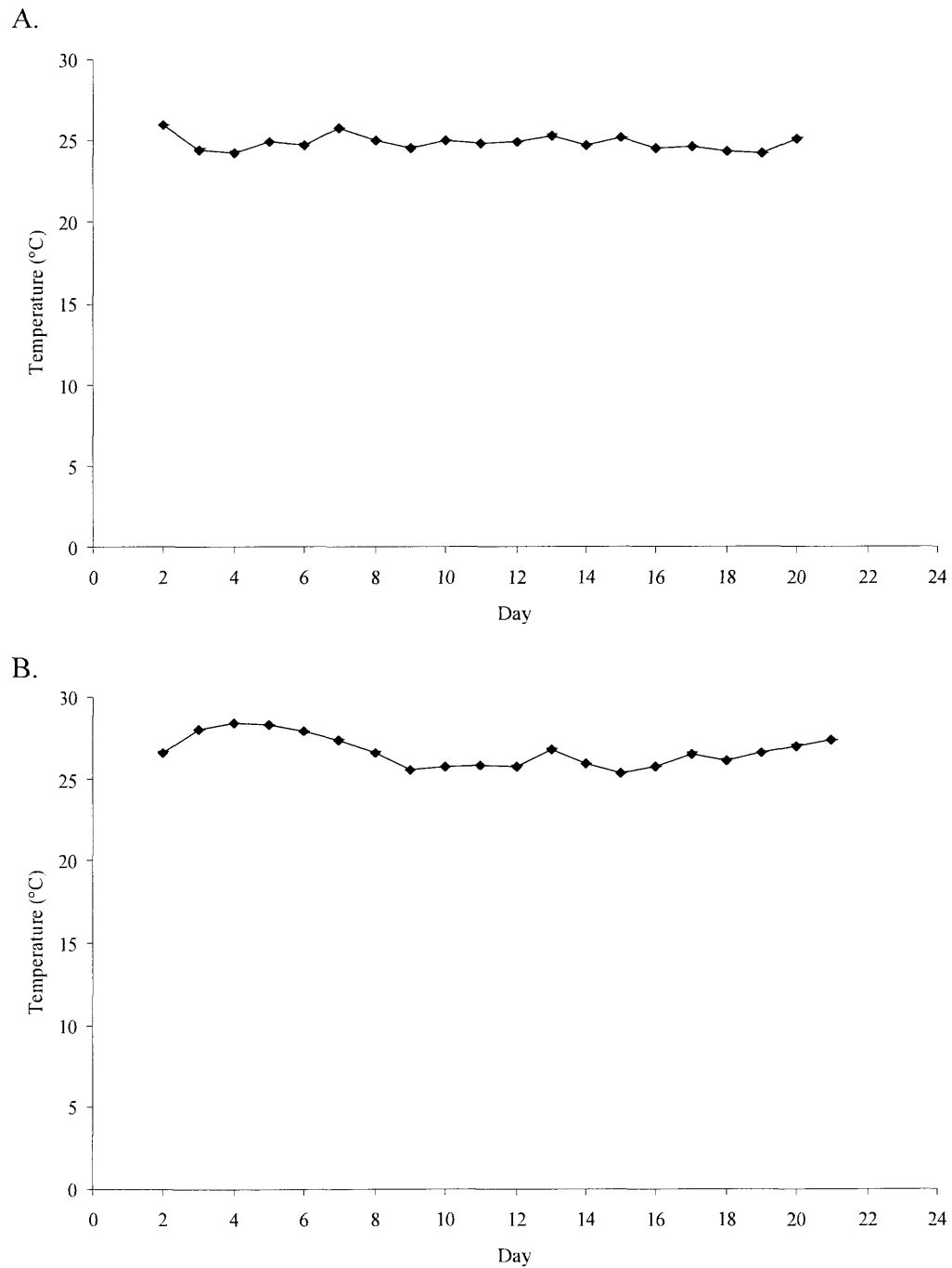
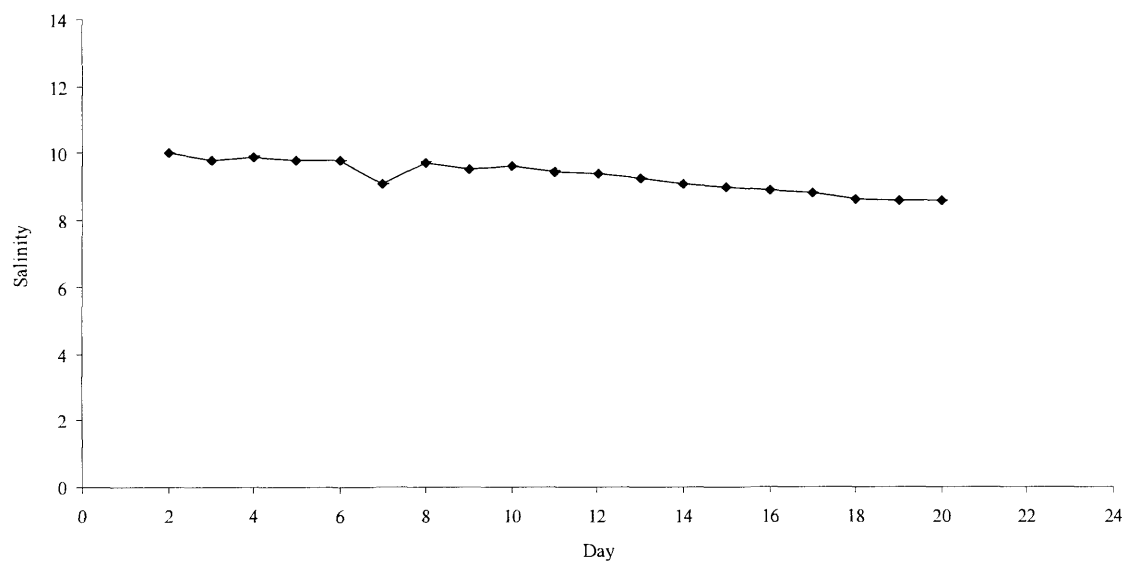


Figure 4.6. Mean daily temperature for flow-through cultures reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. n=6.

A.



B.

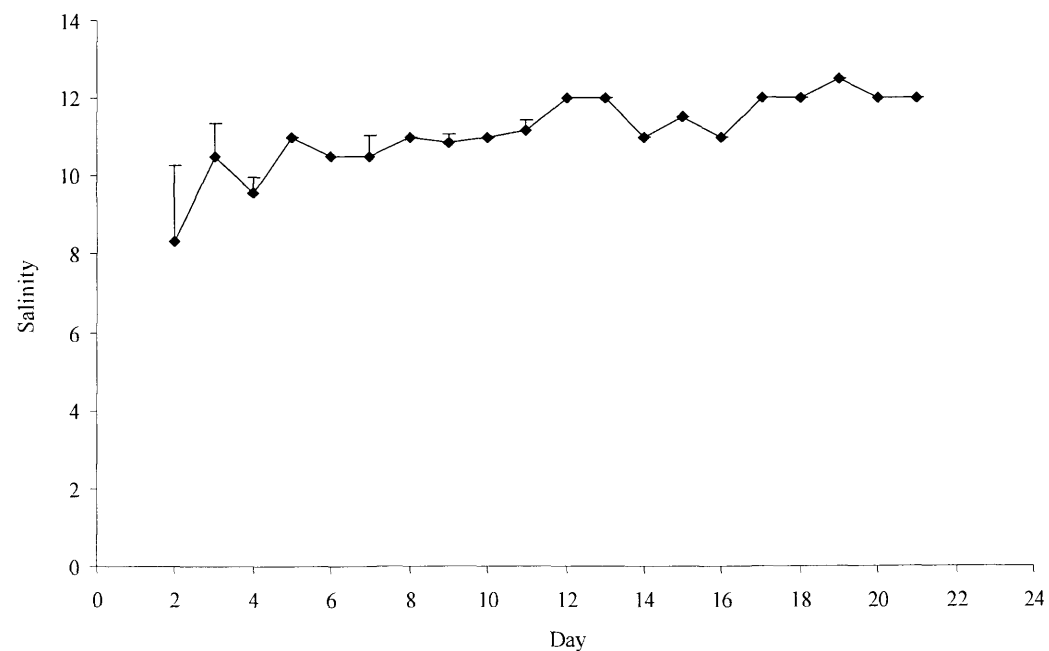


Figure 4.7. Mean daily salinity for flow-through cultures reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. n=6.

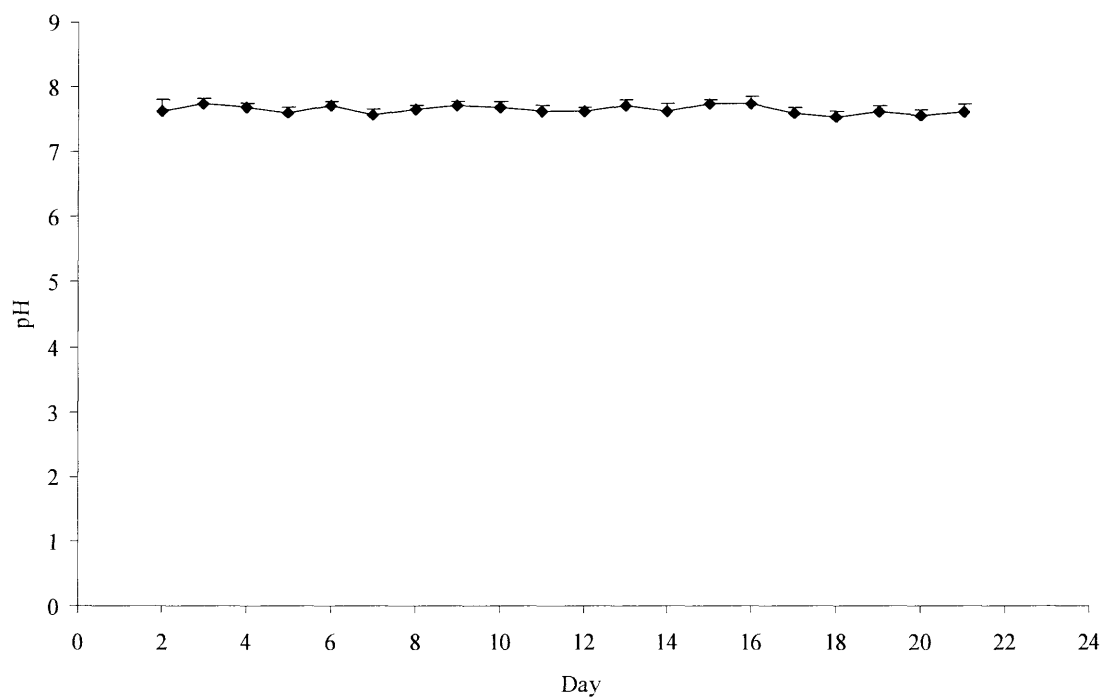
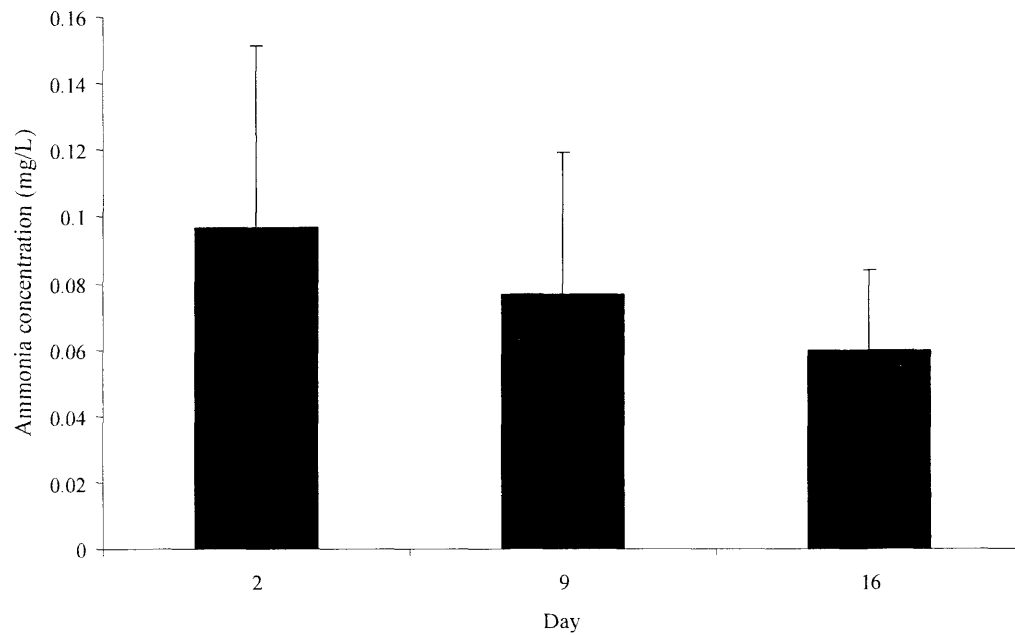


Figure 4.8. Mean daily pH for flow-through culture reared at ten exchanges of water/day. pH data for flow-through cultures reared at five exchanges of water/day is unavailable due to instrument malfunction. Error bars represent +1 sd. n=6.

A.



B.

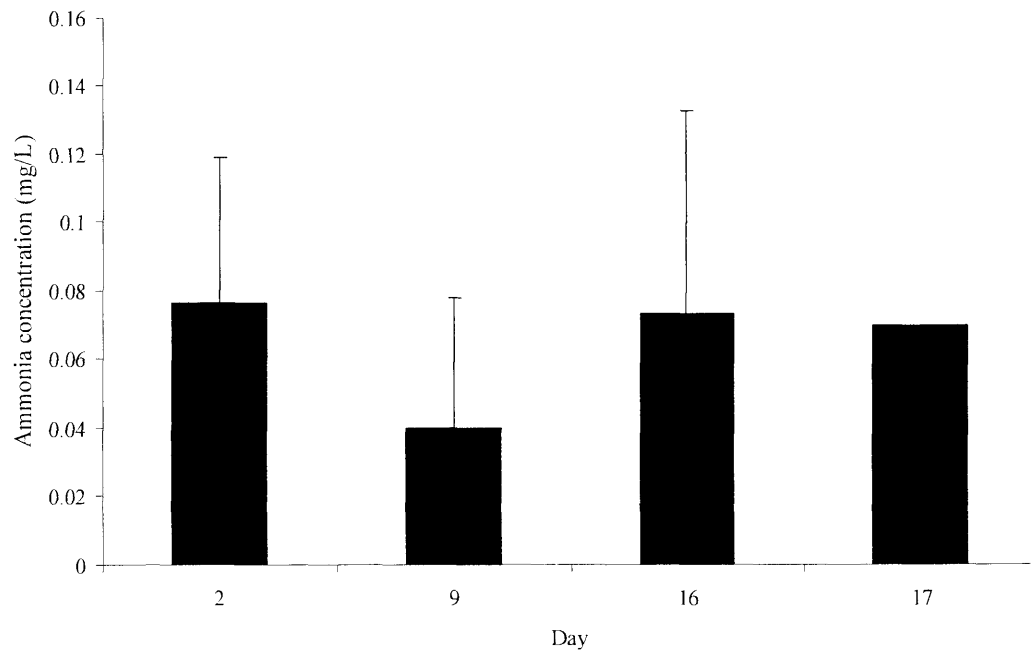


Figure 4.9. Ammonia concentrations (mg/L) for flow-through cultures reared at five exchanges of water/day (A) and ten exchanges of water/day (B). Error bars represent +1 sd. n=6.

Table 4.5. Days and treatment tanks reared at five exchanges of water/day that had bacteria growth on TCBS plates. There was no bacteria growth at ten exchanges of water/day.

Date	Day	Water Temp (°C)	Treatment	Tank	No. colonies/mL
4/28/2011	2	25.9	10/mL	1	100
4/28/2011	2	25.9	10/mL	2	100
4/28/2011	2	25.9	10/mL	6	100

Table 4.6. Coefficient of variations (%) for five variables measured for the controls in the first and second experiments in Chapter Three, reared at 10 larvae/mL and five exchanges of water/day. Age of larvae is designated in the first row. The experiment (first or second) is designated in the second row. n=2

Parameter	2		4		6		9		11		13	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Length	1	1	5	0	8	0.5	10	10	2	4		7
Survival			0.3	32	44	11	29	47	9	75		23
Cell consumption	55	58	83	61	21	56	45	51	1	26		0.9
Growth Efficiency	0.5	65	52	141	38	24	56	64	24	52		28
Cumulative competent harvest									88		8	

Parameter	16		18		20	
	1st	2nd	1st	2nd	1st	2nd
Length		8				
Survival		34				
Cell consumption		45				
Growth Efficiency		25				
Cumulative competent harvest		46		15		1

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